DERIVATIVES OF 1,2-DIHYDRO-7-HYDROXYQUINOLINES CONTAINING FUSED RINGS

FIELD OF THE INVENTION

The invention relates to colored and fluorescent dyes, including reactive dye derivatives, and dye-conjugates; and to their use in staining samples and detecting ligands or other analytes.

BACKGROUND OF THE INVENTION

Fluorescent dyes are known to be particularly suitable for applications in which a highly sensitive detection reagent is desirable. Fluorescent dyes are used to impart both visible color and fluorescence to other materials. As researchers increasingly utilize fluorescent probes as research tools, the ability to select the wavelength of fluorescence becomes more important, particularly asmore multiple-color applications are developed.

A variety of fluorescent dyes have been previously and extensively described, including coumarins, fluoresceins, rhodamines, rhodols, oxazines, carbocyanines, and derivatives thereof. The selection of certain substituents has been shown to be useful in adjusting the spectral properties of such dyes but there have remained regions of the visible spectrum where suitable fluorescent dyes either did not exist, or did not possess particularly favorable properties.

The dyes of the invention incorporate additional fused aromatic or heteroaromatic rings, and exhibit a shift of fluorescence emission to longer wavelength that is typically greater than 20 nm, relative to otherwise structurally similar dyes known in the art. This bathochromic spectral shift yields dyes that are particularly useful for excitation in the wavelength ranges between 400 nm and 600 nm and in particular at greater than 630 nm. Of particular importance are the dyes of the invention that exhibit absorbance maxima

between 530 nm and 650 nm, as they match the principal emission lines of the mercury arc lamp (546 nm), frequency-doubled Nd-Yag laser (532 nm), Kr-ion laser (568 nm, and 647 nm) and HeNe laser (543 nm, 594 nm, and 633 nm).

Fluorescent dyes of the invention with longer wavelength absorption and emission are particularly useful in conjunction with materials of biological origin such as blood, urine, fecal matter, cells and tissues, because background or inherent fluorescence or absorption is less likely to interfere with dye detection. Furthermore, infrared dyes of the invention have enhanced utility in biological systems that are transparent at infrared wavelengths. The long wavelength dyes of the invention also have advantages in use as laser dyes, or in electronics as optical memory elements using relatively low cost illumination sources such as laser diodes.

DESCRIPTION OF DRAWINGS

Figure 1: The excitation and emission spectra of a streptavidin conjugate of Compound 42.

Figure 2: A comparison of the rate of photobleaching between Compound 42 and the fluorescent dye CY-5 in phosphate-buffered saline, as described in Example 54.

Figure 3: The effect of pH on the fluorescence emission of Compound 57, as described in Example 44.

SUMMARY OF THE INVENTION AND DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention describes derivatives of 1,2-dihydro-7-hydroxyquinoline useful for the preparation of a variety of fluorescent dyes and dye derivatives. In the derivatives described herein, the 3,4-double bond of the dihydroquinoline is fused to an aromatic or heteroaromatic ring that is in turn optionally fused to one or more additional aromatic or heteroaromatic rings. The invention also includes dyes that are prepared from the novel synthetic precursors, including, but not limited to, coumarin compounds, rhodamine

compounds, rhodol compounds, triarylmethane compounds, phenoxazine compounds, and their benzo and annelated derivatives. The dyes of the invention optionally possess a reactive group useful for preparing fluorescent conjugates, which conjugates and methods for their preparation and use are described herein.

As used herein, sulfonic acid means either $-SO_3H$, or a salt of sulfonic acid. Also as used herein, carboxylic acid means either -COOH, or a salt of carboxylic acid. Appropriate salts of sulfonic and carboxylic acids include, among others, K^+, Na^+, Cs^+, Li^+, Ca^{2+}, Mg^{2+}, ammonium, alkylammonium or hydroxyalkylammonium salts, or pyridinium salts. Alternatively, the counterion of the sulfonic acid or carboxylic acid may form an inner salt with a positively charged atom on the dye itself, typically a quaternary nitrogen atom.

As used herein, the alkyl portions of substituents such as alkyl, alkoxy, or perfluoroalkyl is optionally saturated, unsaturated, linear or branched.

Where a ring substituent is an aryl substituent, the aryl substituent is defined as an aromatic or polyaromatic moiety containing 1 to 4 aromatic rings (each ring containing 6 conjugated carbon atoms and no heteroatoms) that are optionally fused to each other or bonded to each other by carbon-carbon single bonds. Aryl substituents are bound by a single bond and are optionally substituted as described below.

Specific examples of aryl substituents include, but are not limited to, substituted or unsubstituted derivatives of phenyl, biphenyl, *o*-, *m*-, or *p*-terphenyl, 1-naphthyl, 2-naphthyl, 1-, 2-, or 9-anthryl, 1-, 2-, 3-, 4-, or 9-phenanthrenyl and 1-, 2- or 4-pyrenyl. Preferred aryl substituents are phenyl, substituted phenyl, naphthyl or substituted naphthyl.

Where a ring substituent is a heteroaryl substituent, it is defined as a 5- or 6-membered heteroaromatic ring that is optionally fused to an additional six-membered aromatic ring(s), or is fused to one 5- or 6-membered heteroaromatic ring. The heteroaromatic rings contain at least 1 and as many as 3 heteroatoms that are selected

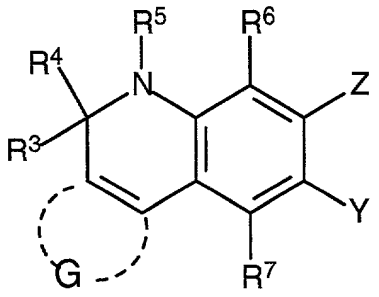
from the group consisting of O, N or S in any combination. The heteroaryl substituent is bound by a single bond, and is optionally substituted as defined below.

Specific examples of heteroaryl moieties include, but are not limited to, substituted or unsubstituted derivatives of 2- or 3-furanyl; 2- or 3-thienyl; N-, 2- or 3-pyrrolyl; 2- or 3-benzofuranyl; 2- or 3-benzothienyl; N-, 2- or 3-indolyl; 2-, 3- or 4-pyridyl; 2-, 3- or 4-quinolyl; 1-, 3-, or 4-isoquinolyl; 2-, 4-, or 5-(1,3-oxazolyl); 2-benzoxazolyl; 2-, 4-, or 5-(1,3-thiazolyl); 2-benzothiazolyl; 3-, 4-, or 5-isoxazolyl; N-, 2-, or 4-imidazolyl; N-, or 2-benzimidazolyl; 1- or 2-naphthofuranyl; 1- or 2-naphthothienyl; N-, 2- or 3-benzindolyl; 2-, 3-, or 4-benzoquinolyl; 1-, 2-, 3-, or 4-acridinyl. Preferred heteroaryl substituents include substituted or unsubstituted 4-pyridyl, 2-thienyl, 2-pyrrolyl, 2-indolyl, 2-oxazolyl, 2-benzothiazolyl or 2-benzoxazolyl.

The aryl and heteroaryl substituents described herein are unsubstituted or optionally and independently substituted by H, halogen, cyano, sulfonic acid, carboxylic acid, nitro, alkyl, perfluoroalkyl, alkoxy, alkylthio, amino, monoalkylamino, dialkylamino or alkylamido.

Although typically not shown for the sake of clarity, any overall positive or negative charges possessed by any of the compounds of the invention are balanced by a necessary counterion or counterions. Where the compound of the invention is positively charged, the counterion is typically selected from, but not limited to, chloride, bromide, iodide, sulfate, alkanesulfonate, arylsulfonate, phosphate, perchlorate, tetrafluoroborate, tetraarylboride, nitrate and anions of aromatic or aliphatic carboxylic acids. Where the compound of the invention is negatively charged, the counterion is typically selected from, but not limited to, alkali metal ions, alkaline earth metal ions, transition metal ions, ammonium or substituted ammonium ions. Preferably, any necessary counterion is biologically compatible, is not toxic as used, and does not have a substantially deleterious effect on biomolecules. Counterions are readily changed by methods well known in the art, such as ion-exchange chromatography, or selective precipitation.

The synthetic precursors have the formula:



G represents the atoms necessary to form a 5- or 6-membered aromatic or heteroaromatic fused ring, that is optionally substituted one or more times by sulfonic acid, carboxylic acid, or C_1-C_6 alkyl or alkoxy that is optionally substituted by carboxylic acid, sulfonic acid, or halogen; or by an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, azido, carboxylic acid, sulfonic acid, or halomethyl. The fused ring G optionally contains one or two additional fused aromatic or heteroaromatic rings that are optionally sulfonated one or more times.

Suitable examples of fused aromatic rings include, but are not limited to, substituted or unsubstituted derivatives of benzenes, naphthalenes, anthracenes, phenanthracenes, or pyrenes. Preferred aromatic rings include substituted or unsubstituted benzene or naphthalenes.

Suitable examples of fused heteroaromatic rings include, but are not limited to, furans, thiophenes, pyrrols, benzofurans, benzothiophenes, indoles, pyridines, quinolines, isoquinolines, oxazoles, benzoxazoles, thiazoles, benzothiazoles, isoxazoles, imidazoles, benzimidazoles, naphthofurans, naphthothiophenes, benzindoles, benzoquinolines, or acridines. Preferred fused heteroaromatic rings include substituted or unsubstituted one or more of R^1, R^2, and R^6 is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, azido, carboxylic acid, sulfonic

acid, or halomethylpyridines, thiophenes, pyrroles, indoles, oxazoles, benzothiophenes, and benzoxazoles.

R^6 is H, cyano, halogen, carboxylic acid, or sulfonic acid, or a C_1-C_6 alkyl or C_1-C_6 alkoxy that is optionally substituted by carboxylic acid, sulfonic acid, or halogen. Additionally R^6 is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, azido, carboxylic acid, sulfonic acid, or halomethyl.

The substituents R^3 and R^4 are independently H, or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, hydroxy, or halogen. Alternatively one or both of R^3 and R^4 is independently an aromatic or heteroaromatic ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. In another aspect of the invention, R^3 and R^4, taken in combination, form a 5- or 6-membered ring that optionally contains 1 or 2 heteroatoms. Typically, where R^3 and R^4 form a ring, it is an alicyclic ring. R^3 and R^4 are each typically alkyl, and preferably R^3 and R^4 are methyl.

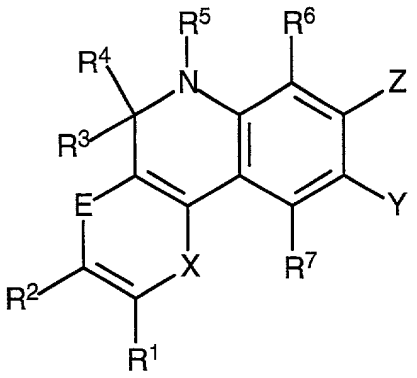
The R^5 substituent is H, methyl, carboxymethyl, or a C_2-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. Alternatively, R^5 is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. In another aspect of the invention, R^4 taken in combination with R^5, or R^5 taken in combination with R^6, forms a 5- or 6-membered alicyclic ring.

The R^7 substituent is hydrogen, C_1-C_6 alkyl, or C_1-C_6 alkoxy. Typically, R^7 is hydrogen.

Y is optionally H, OH, NH_2, NO, -(CO)-R^9, or -(CO)-O-R^{10}, where R^9 and R^{10} are H, C_1-C_6 alkyl, a substituted or unsubstituted aryl or heteroaryl ring system having 1-2 rings.

Z is optionally H, OH, NHR^{17}, SH, or $C(CR^{11}R^{12})_2OH$; where R^{17} is a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. The R^{11} and R^{12} substituents are independently C_1-C_6 alkyls that are themselves optionally substituted by carboxylic acid, sulfonic acid, or halogen, or R^{11} and R^{12} taken in combination form a 5- or 6-membered alicyclic ring. Preferably R^{11} and R^{12} are each methyl.

In one embodiment, the synthetic precursor has the formula



where R^1 and R^2 are independently selected from H, cyano, halogen, carboxylic acid, or sulfonic acid, or one or more of R^1 and R^2 may be a C_1-C_6 alkyl or alkoxy that is optionally substituted by carboxylic acid, sulfonic acid, or halogen. Additionally one or more of R^1 and R^2 is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, azido, carboxylic acid, sulfonic acid, or halomethyl. In another aspect of the invention, R^1 in combination with R^2 forms a fused aromatic or heteroaromatic ring that is optionally sulfonated one or more times. In another aspect of the invention, R^2 taken in combination with R^3 forms a 5- or 6-membered alicyclic ring.

One of X and E is selected from O, S, NR^8, or $CR^{1'}=CR^{2'}$, and the other is absent. The substituent R^8 is H, methyl, carboxymethyl, or a C_2-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. The substituents $R^{1'}$ and $R^{2'}$ are as defined above for R^1 and R^2. In one embodiment, one of X and E is S or O. In another

embodiment, X is O, S, NR^8, or $CR^{1'}=CR^{2'}$, and E is absent. In yet another embodiment, E is O or S, and X is absent.

Y is optionally H, OH, NH_2, NO, -(CO)-R^9, or -(CO)-O-R^{10}, where R^9 and R^{10} are H, C_1-C_6 alkyl, a substituted or unsubstituted aryl or heteroaryl ring system having 1-2 rings.

Z is optionally H, OH, NHR^{17}, SH, or $C(CR^{11}R^{12})_2OH$; where R^{17} is a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. The R^{11} and R^{12} substituents are independently C_1-C_6 alkyls that are themselves optionally substituted by carboxylic acid, sulfonic acid, or halogen, or R^{11} and R^{12} taken in combination form a 5- or 6-membered alicyclic ring.

The precursors of the invention are optionally substituted by a covalently bound reactive group (-L-R_X) or conjugated substance (-L-S_C) as will be described below. In this embodiment, one or more of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8 is -L-R_x or -L-S_C, or where R^1 taken in combination with R^2 forms a fused aromatic or heteroaromatic ring, the resulting ring is substituted by -L-R_x or -L-S_C.

Condensation reactions of the novel precursors

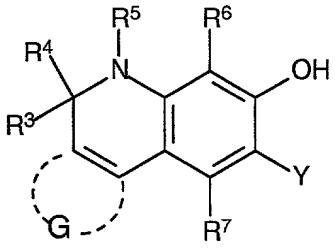
A variety of useful and novel dyes are readily prepared using the precursors of the invention. The dyes of the invention are prepared by condensing a first precursor (a precursor of the invention) with a second precursor, simultaneous with, before, or after reaction with a third precursor. The types of precursors and possible dye products are described in greater detail below. The utilization of the precursors of the invention in these syntheses results in dyes whose absorption is shifted to substantially longer wavelengths, compared to structurally related dyes that do not possess the additional fused ring systems.

Derivatives of 3-aminophenol are valuable synthetic intermediates in the synthesis of a variety of dyes. Numerous classes of commercially important dyes may be prepared with appropriately substituted 3-minophenols using well known synthetic strategies. In

particular, 3-aminophenols have been utilized in the preparation of coumarin dyes (U.S. Patent No. 5,696,157 to Wang et al. (1997), incorporated by reference), rhodol dyes (U.S. Patent No. 5,227,487 to Haugland et al. (1993), incorporated by reference), rhodamine dyes (Copending application serial no. 08/935,963, titled "Sulfonated Xanthene Derivatives", filed 9/23/97, incorporated by reference), and oxazine dyes. 3-Aminophenols have also been utilized to prepare annelated rhodol dyes (seminaphthorhodafluor dyes) commercially available under the trademark SNARF (U.S. Patent No. 4,945,171 to Haugland et al. (1990), incorporated by reference).

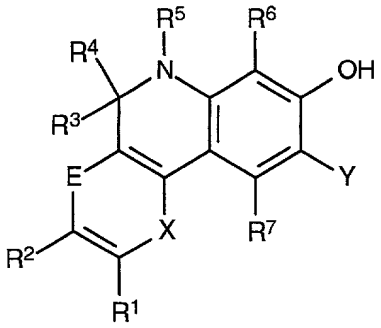
Typically the condensation reaction utilizing the 3-aminophenol derivatives of the invention is acid-catalyzed, and utilizes a first precursor, a second precursor, and optionally utilizes a third precursor. This condensation occurs in the presence or absence of various acid catalysts (such as zinc chloride, *p*-toluenesulfonic acid, sulfuric acid, or methanesulfonic acid). An aqueous workup, typically followed by column chromatography, yields the desired dye.

The first precursor is a derivative of 3-aminophenol of the invention having the formula



wherein G, R^3, R^4, R^5, and R^6 are as defined earlier. R^7 is hydrogen or C_1-C_6 alkyl. Y is optionally H, OH, NO, -(CO)-R^9, or -(CO)-O-R^{10}, where R^9 and R^{10} are H, C_1-C_6 alkyl, a substituted or unsubstituted aryl or heteroaryl ring system having 1-2 rings.

Typically, the first precursor has the formula



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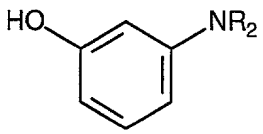
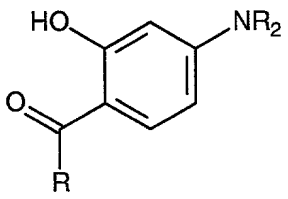
wherein E, X, R^1, R^2, R^3, R^4, R^5, and R^6 are as defined earlier. R^7 is hydrogen or C_1-C_6 alkyl. Y is optionally H, OH, NO, -(CO)-R^9, or -(CO)-O-R^{10}, where R^9 and R^{10} are H, C_1-C_6 alkyl, a substituted or unsubstituted aryl or heteroaryl ring system having 1-2 rings.

Where Y is NO, the first precursor is a 6-nitroso-3-aminophenol derivative. Where Y is -(CO)-R^9, the first precursor is a 6-acyl-3-aminophenol derivative.

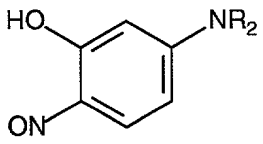
The second precursor is permitted to be a 3-aminophenol (to yield a rhodamine), a resorcinol (to yield a rhodol), a 1,6-naphthalenediol (to yield an annelated rhodol), a 6-amino-1-naphthol (to yield an annelated rhodamine), an alpha-methylene acid, an alpha methylene ester, an alpha-methylene nitrile, or a beta-keto ester (all of which yield coumarins). Generic examples of each of these types of precursors are given below, although a variety of substitutions and variations are permissible without effectively diminishing the efficacy of the condensation reaction:



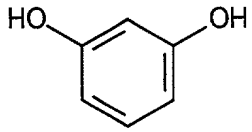
a 3-aminophenol



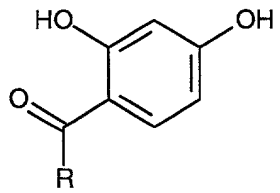
a 6-acyl-3-aminophenol



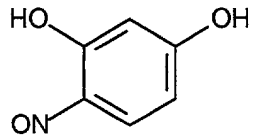
a 6-nitroso-3-aminophenol



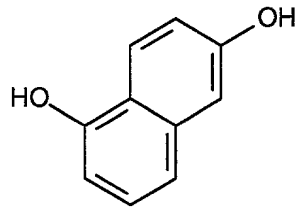
a resorcinol



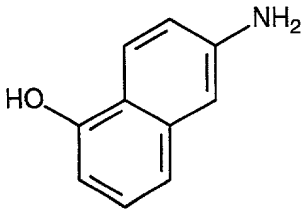
a 4-acylresorcinol



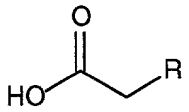
a 4-nitrosoresorcinol



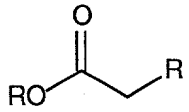
a 1,6-naphthalenediol



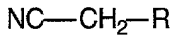
a 6-amino-1-naphthol



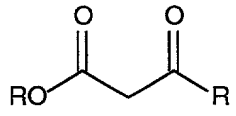
an alpha-methylene acid



an alpha-methylene ester



an alpha-methylene nitrile



a beta-keto ester

The condensation reaction optionally incorporates a third precursor. The third precursor is typically an aldehyde or an acid, including diacids, acid anhydrides, esters, acid halides and similar acylating agents derived from acids. Examples of aldehydes include formaldehyde, lower aliphatic and alicyclic aldehydes, heterocyclic aldehydes and especially benzaldehydes and less preferably naphthaldehydes. Most commonly the third precursor is an aliphatic or aromatic diacid, or a cyclic anhydride of an aliphatic or aromatic diacid. Particularly preferred third precursors are derivatives of succinic acid, glutaric acid, phthalic acid, or ortho-sulfobenzoic acid.

In those condensation reactions where the third precursor is present, the reaction of

the first, second and third precursors is typically a stepwise reaction. The third precursor reacts with either the first or second precursor to form an intermediate condensation product, which then reacts with the remaining precursor.

Where the first and second precursors are the same, the resulting dye is symmetric. Typically, where symmetric dyes are prepared, the condensation includes a third precursor, as described above. Commonly one molecule of a substituted or unsubstituted phthalic acid or anhydride or a substituted or unsubstituted ortho-sulfobenzaldehyde or ortho-sulfobenzoic acid (the third precursor) is condensed with at least two molecules of a 3-aminophenol of the invention (first and second precursor, which are the same) to produce a symmetric rhodamine dye. Less commonly a 3-aminophenol of the invention (first precursor) and a resorcinol or a different 3-aminophenol (second precursor) is condensed with a substituted or unsubstituted phthalic acid or o-sulfobenzoic acid derivative (third precursor) to produce rhodol or asymmetric rhodamine dyes of the invention.

Where the first precursor is a 6-nitroso-3-aminophenol, and the second precursor is a resorcinol or a 3-aminophenol, the resulting dye is an oxazine. Alternatively, the second precursor is nitroso-substituted, such as a 4-nitrosoresorcinol or a 6-nitroso-3-aminophenol. In either case, the resulting product is an oxazine dye.

To prepare coumarin dyes, a first precursor that is a 6-acyl-3-aminophenol is reacted with a second precursor that is an alpha-methylene acid, ester, or nitrile to yield a 3-substituted coumarin, the nature of the 3-substituent of the resulting coumarin dye being dependent upon the 6-acyl moiety on the first precursor. 4-Substituted coumarin dyes are prepared using a first precursor that is a 3-aminophenol of the invention with a second precursor that is a beta-keto ester. In this instance, the nature of the 4-substituent is dependent upon beta-keto ester selected.

Selected condensation reactions of the invention are outlined in Table 1, below.

Table 1.

First Precursor	Second Precursor	Third Precursor	Product Dye	Examples
a 3-aminophenol	a 3-aminophenol	an aldehyde, acid, diacid, or anhydride	a symmetric or asymmetric rhodamine	18, 29, 30, 32, 33, 36
a 3-aminophenol	a 6-acyl-3-aminophenol	none		
a 6-acyl-3-aminophenol	a 3-aminophenol	none		
a 3-aminophenol	a resorcinol	an aldehyde, acid, diacid, or anhydride	a rhodol	35
a 6-acyl-3-aminophenol	a resorcinol	none		
a 3-aminophenol	a 4-acylresorcinol	none		
a 3-aminophenol	a 4-nitrosoresorcinol, or a 6-nitroso-3-aminophenol	none	an oxazine	21
a 6-nitroso-3-aminophenol	a resorcinol, or a 3-aminophenol	none		
a 3-aminophenol	a 1,6-naphthalenediol, or 6-amino-1-naphthol	an aldehyde, acid, diacid, or anhydride	an annelated rhodol or rhodamine	38
a 6-nitroso-3-aminophenol	a 1,6-naphthalenediol, or a 6-amino-1-naphthol	none	an annelated oxazine	
a 6-acyl-3-aminophenol	an alpha-methylene acid, ester or nitrile	none	a 3-substituted coumarin	20
a 3-aminophenol	a beta-keto ester	none	a 4-substituted coumarin	

In addition to the rhodamine, annelated rhodamine, rhodol, annelated rhodol, oxazine, annelated oxazine and coumarin dyes whose synthesis and properties is described in this invention, first precursors wherein Z is not hydroxy are useful synthetic precursors a variety of analogous classes of dyes. Precursors wherein Z is SH are useful for preparing thiocoumarins, thiorhodamines, thiooxazines and thiorhodols (for example as in EP 0 330 444 to Chen et al., (1989)) and their annelated versions. Precursors wherein Z is NHR^{17} are useful for preparing "azacoumarins" (carbostyryls), "azarhodamines" (acridines), "azarhodols" (acridines), "azaoxazines" (phenazines) and their annelated versions. Precursors wherein Z is $C(CR^{11}R^{12})_2OH$ are useful for preparing carbazine dyes and their analogs. Precursors wherein Z is H are useful for preparing triarylmethane dyes and their analogs (Example 34).

In one aspect of the invention, the resulting dye is sulfonated. Sulfonation can be done subsequent to the condensation reaction (as in Example 23), or one or more precursors may be sulfonated prior to formation of the dye (as for Compound 34 in Example 18).

Sulfonation of dyes is typically carried out by stirring the dye in fuming sulfuric acid (20-30% SO_3 content) or concentrated sulfuric acid at an appropriate temperature. Mono-sulfonation of rhodol dyes is carried out by stirring the appropriate rhodol dye in fuming sulfuric acid at 0 °C for several hours. Bis-sulfonation of rhodols at both the 4′- and 5′-positions, if available, is achieved by stirring the dye in fuming sulfuric acid at room temperature for several hours. Sulfonation of most rhodamine or rosamine dyes at the 4′- and 5′-positions, if available, is carried out with fuming sulfuric acid at 0 °C; the sulfonation is usually complete as soon as a homogeneous solution is achieved during stirring.

Post-condensation modification of xanthene-based dyes is well known. For example, the xanthene portion of the dye can be halogenated by treatment with the appropriate halogenating agent, such as liquid bromine. Xanthenes containing unsaturated fused rings can be hydrogenated to the saturated derivatives. When trimellitic anhydride,

nitrophthalic anhydride, or their derivatives are used in the dye synthesis, two isomeric carboxylates or nitro derivatives are typically formed. These isomers are separated or, in most cases, used as the mixture of isomers. The reduced derivatives of xanthylium dyes are typically prepared by chemical reduction of the xanthenone portion with zinc dust, borohydride in organic solvents, or by catalytic hydrogenation. The amino and hydroxyl groups of the dyes of the invention can be acylated or alkylated to yield amides, esters and ethers. Selected amide, ether and ester derivatives of the invention possess potential utility as chromogenic or fluorogenic enzyme substrates.

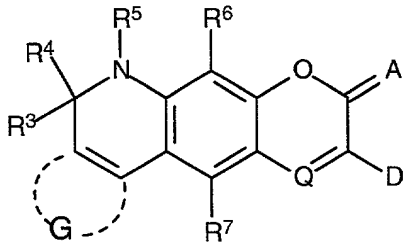
The selection of an appropriate polyhalogenated phthalic acid derivative or benzaldehyde in the condensation of the xanthylium dye results in a dye having a tetra- or pentachlorinated or tetra- or pentafluorinated phenyl ring at the 9-position. These polyhaloaryl substituted dyes have been shown to react with thiols via a displacement reaction, and thereby provide a facile method of introducing additional reactive groups (Example 19; and as discussed by Gee, et al. TET. LETT. **37**, 7905 (1996)).

The dihydroxanthene and xanthylium versions of the dyes of the invention are freely interconvertible by well-known oxidation or reduction reagents, including borohydrides, aluminum hydrides, hydrogen/catalyst, and dithionites. A variety of oxidizing agents mediate the oxidation of dihydroxanthenes, including molecular oxygen in the presence or absence of a catalyst, nitric oxide, peroxynitrite, dichromate, triphenylcarbenium and chloranil. The xanthenes are also oxidized by enzyme action, including horseradish peroxidase in combination with peroxides. Dihydroxanthene dye precursors may also be oxidized inside certain living cells, yielding the corresponding dye compound.

Examples of synthetic strategies for selected dyes of the invention, as well as their characterization, synthetic precursors, conjugates and method of use are provided in the examples below.

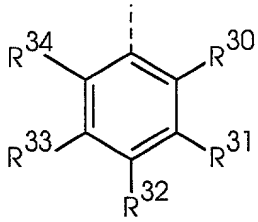
Selected Dye Embodiments

In one aspect of the invention, the dyes of the invention that result from the condensation reaction with the precursors described above have the formula



wherein G, R^3, R^4, R^6, and R^7 are as described above for the novel precursors of the invention.

The Q moiety is N or CR^{28}, wherein R^{28} is H, F, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alcohol. Alternatively R^{28} is a C_1-C_6 alkyl that is optionally substituted one or more times by carboxylic acid, sulfonic acid, amino, or halogen. In another preferred embodiment, Q is CR^{28} where R^{28} has the formula

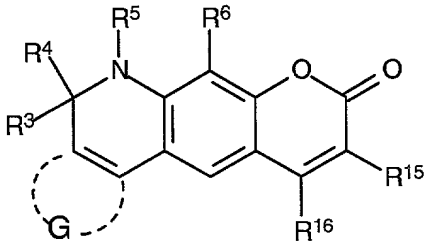


where the substituents R^{30}, R^{31}, R^{32}, R^{33} and R^{34} are independently H, F, Cl, Br, I, sulfonic acid, carboxylic acid, CN, nitro, hydroxy, azido, amino, hydrazino, C_1-C_{18} alkyl, C_1-C_{18} alkoxy, C_1-C_{18} alkylthio, C_1-C_{18} alkanoylamino, C_1-C_{18} alkylaminocarbonyl, C_2-C_{36} dialkylaminocarbonyl, C_1-C_{18} alkyloxycarbonyl, or C_7-C_{18} arylcarboxamido. Where any of the R^{30}, R^{31}, R^{32}, R^{33} and R^{34} substituents are alkyl, alkoxy, alkylthio, alkanoylamino, alkylaminocarbonyl, dialkylaminocarbonyl, alkyloxycarbonyl, or arylcarboxamido, the alkyl or aryl portions of the substituents are optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxylic acid, a carboxylic acid ester of a C_1-C_6 alcohol, sulfonic acid,

amino, alkylamino, dialkylamino or alkoxy (the alkyl portions of each having 1-6 carbons). Alternatively, one pair of adjacent substituents R^{31} and R^{32}, R^{32} and R^{33} or R^{33} and R^{34}, when taken in combination, form a fused 6-membered aromatic ring that is optionally further substituted by carboxylic acid. Alternatively, one or more of R^{30}, R^{31}, R^{32}, R^{33} and R^{34} is -L-R_x or -L-S_C, as described below.

In one aspect of the invention, the resulting dye is a coumarin. In this embodiment, A is O, and D is a monovalent substituent. In another aspect of the invention, A and D, when taken in combination, form an aromatic or heteroaromatic ring system having 1-3 additional rings, where the ring system is optionally substituted.

In one aspect of the invention, the dye of the invention is a coumarin dye having the formula

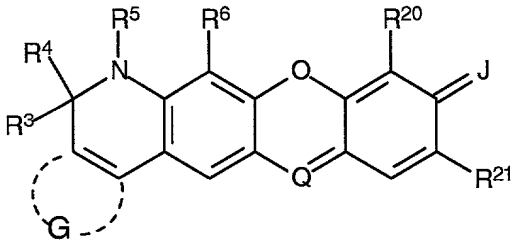


where GR^3, R^4, R^5, and R^6 are as defined above.

The substituents R^{15} and R^{16} are independently hydrogen, cyano, nitro, halogen, carboxylic acid, sulfonic acid, or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, or halogen. Alternatively, one or more of R^{15} and R^{16} is an aromatic or heteroaromatic ring system having 1-2 fused rings that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. In another aspect of the invention, one of R^{15} and R^{16} is -L-R_x or -L-S_C.

In one aspect of the invention, one of R^{15} and R^{16} is nonhydrogen. In another aspect of the invention, both R^{15} and R^{16} are nonhydrogen. In one aspect of the invention, R^{16} is H. In another aspect of the invention, R^{16} is chloromethyl or bromomethyl. In yet another aspect of the invention, R^5 is not hydrogen, and R^6 is methyl or C_1-C_6 alkyl optionally substituted by sulfonic acid or carboxylic acid. Where one of R^{15} or R^{16} is a heteroaromatic ring system, it is typically a benzothiazole.

In another aspect of the invention, the dye of the invention is a derivative of a xanthene or oxazine dye having the formula



where G, R^3, R^4, R^5, and R^6 are as defined above.

The substituents R^{20} and R^{21} are hydrogen, cyano, halogen, carboxylic acid, sulfonic acid, or a C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted by carboxylic acid, sulfonic acid, or halogen. Alternatively, one or both of R^{20} and R^{21} is an aromatic or heteroaromatic ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. Either or both of R^{20} and R^{21} is optionally -L-R_x; or -L-S_C.

The J moiety is O or $NR^{37}R^{38}$, where R^{37} and R^{38} are independently H or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. Alternatively, one or more of R^{37} and R^{38} is -L-R_x or -L-S_C.

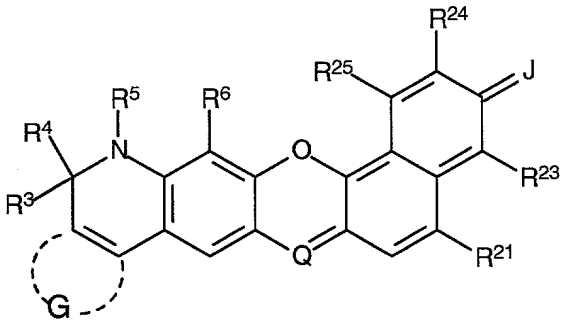
Alternatively one of R^{37} and R^{38} is an aryl or heteroaryl ring, or R^{37} when taken in combination with R^{38} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a

morpholine, a pyrrolidine or a piperazine, wherein the heterocycle is optionally substituted by methyl, carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl.

In yet another alternative, R^{37} taken in combination with R^{20}, or R^{38} taken in combination with R^{21}, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more sulfonic acids, or by one or more C_1-C_6 alkyl groups that are optionally substituted by sulfonic acid.

Q is N or CR^{28}, as described above.

In another aspect of the invention, the dye of the invention is a seminaphthorhodafluor derivative having the formula



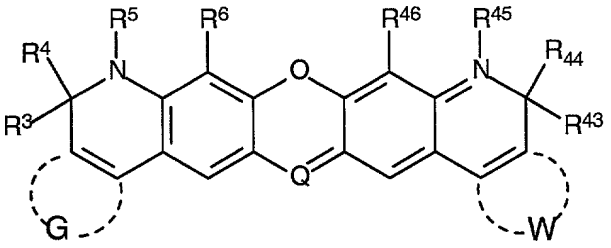
where G, R^3, R^4, R^5, R^6, and Q are as defined above.

The substituents R^{21}, R^{23}, R^{24}, and R^{25} are hydrogen, cyano, nitro, halogen, carboxylic acid, or sulfonic acid, or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, or halogen. Alternatively one or more of R^{21}, R^{23}, R^{24}, and R^{25} is an aromatic or heteroaromatic ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. Additionally, one or more of R^{21}, R^{23}, R^{24}, and R^{25} is -L-R_x or -L-S_C.

The J moiety is O or $NR^{37}R^{38}$, where R^{37} and R^{38} are independently H or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. Alternatively, one or more of R^{37} and R^{38} is -L-R_x or -L-S_C.

Derivatives of seminaphthorhodafluor are typically useful as fluorescent pH indicators (see Example 44).

In yet another aspect of the invention, the dye product incorporates two precursor compounds of the invention to yield a rhodamine dye having the formula



where G, R^3, R^4, R^5, R^6, and Q are as defined above.

The substituent R^{46} is H, cyano, halogen, carboxylic acid, sulfonic acid, or C_1-C_6 alkyl or alkoxy that is optionally substituted by carboxylic acid, sulfonic acid, or halogen. Alternatively R^{46} is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, azido, carboxylic acid, sulfonic acid, or halomethyl. R^{46} may also be -L-R_x or -L-S_C.

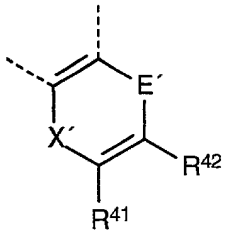
The substituents R^{43} and R^{44} are independently H, or a C_1-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, hydroxy, or halogen. Alternatively one or both of R^{43} and R^{44} is independently an aromatic or heteroaromatic ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. R^{43} and R^{44} are each typically alkyl, and preferably R^{43} and R^{44} are methyl. In another aspect of the invention, R^{43} taken in

combination with R^{44}, forms a 5- or 6-membered alicyclic ring. Alternatively, R^{43} and R^{44} are -L-R_x or -L-S_C.

The R^{45} substituent is H, methyl, carboxymethyl, or a C_2-C_6 alkyl that is optionally substituted by carboxylic acid, sulfonic acid, amino, or halogen. Alternatively, R^{45} is an aryl or heteroaryl ring that is optionally substituted one or more times by C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, cyano, halogen, carboxylic acid, sulfonic acid, or halomethyl. In another aspect of the invention, R^{44} taken in combination with R^{45}, or R^{45} taken in combination with R^{46}, forms a 5- or 6-membered alicyclic ring. Alternatively, R^{45} is -L-R_x or -L-S_C.

The fused ring W is a 5- or 6-membered aromatic or heteroaromatic fused ring that has the same parameters defined for G above, and is optionally the same as or different from G.

In one embodiment, the fused ring W has the formula



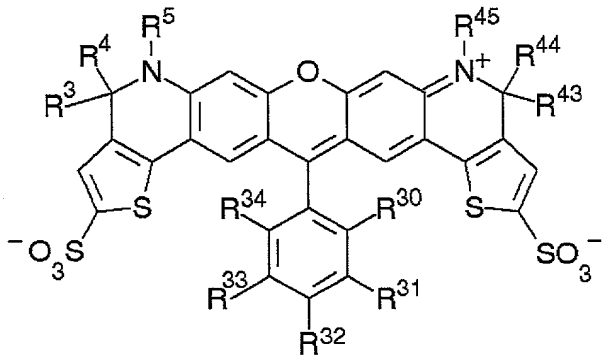
where X´and E´ are defined as for X and E above, and R^{41} is defined as R^1 above, and R^{42} is defined as R^2 above.

Typically, in this embodiment, the dye is fully symmetrical. That is, G and W are the same. In particular, X = X´, E = E´, $R^1 = R^{41}$, and $R^2 = R^{42}$. In a fully symmetrical dye, R^3 and R^{43} are the same, R^4 and R^{44} are the same, R^5 and R^{45} are the same, and R^6 and R^{46} are

the same. In another aspect of the invention, R^3, R^4, R^{43}, and R^{44} are each methyl. In one preferred embodiment, each X is S, and each R^1 is sulfonic acid.

In one embodiment, the dyes of the invention are derivatives of 3- and/or 6-amino xanthenes that are substituted at one or more amino nitrogen atoms by an aromatic or heteroaromatic ring system (i.e., one or more of R^5, R^{37}, and R^{38} is an aromatic or heteroaromatic ring). N-aryl rhodamines have been shown to be efficient and minimally fluorescent energy acceptors (See application serial no. 09/556,464; filed April 21, 2000 by Haugland et al., incorporated by reference), and such compounds are useful in any application where a quenching energy acceptor is useful, particularly in applications utilizing fluorescence resonance energy transfer (FRET). In a preferred embodiment, one or more of R^5, R^{37} and R^{38} is phenyl.

In another embodiment of the invention, the dyes have the formula:



wherein R^3, R^4, R^5, R^{43}, R^{44} , and R^{45} are independently methyl or ethyl;

R^{30} is sulfonic acid or carboxylic acid;

R^{31} and R^{34} are independently H, F, or Cl

one of R^{32} and R^{33} is H, F, or Cl, and the other of R^{32} and R^{33} is -L-R_x or -L-S_c,

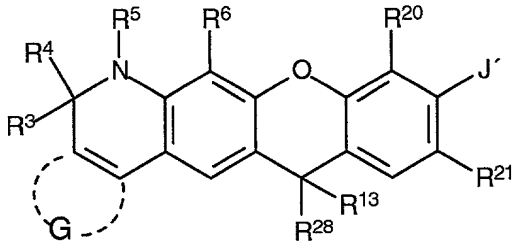
wherein L is a covalent linkage of the formula $-S(CH_2)_aCOO(CH_2)_b-$ or the formula $-S(CH_2)_aCONH(CH_2)_b-$

wherein a is an integer between 0 and 10, and b is an integer between 0 and 10 provided that a and b are not both 0; and R_x and S_c are as defined above. Preferably, R_x , where present, is a carboxylic acid, an activated ester of a carboxylic acid, a haloacetamide,

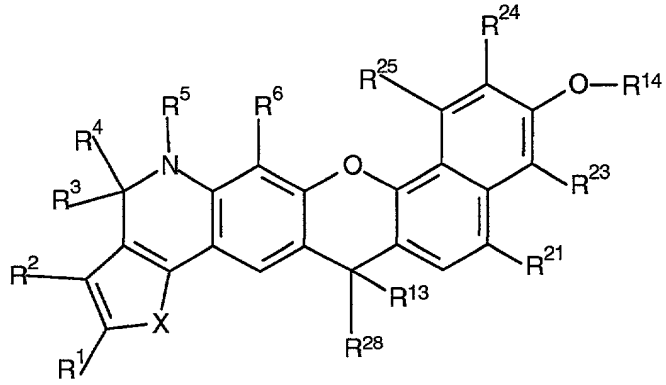
a hydrazine, an isothiocyanate, a maleimide group, or a reactive platinum complex.; and S_c, where present, is an amino acid, a peptide, a protein, an ion-complexing moiety, a nucleoside, a nucleotide, an oligonucleotide, or a nucleic acid.

Reduced Dyes

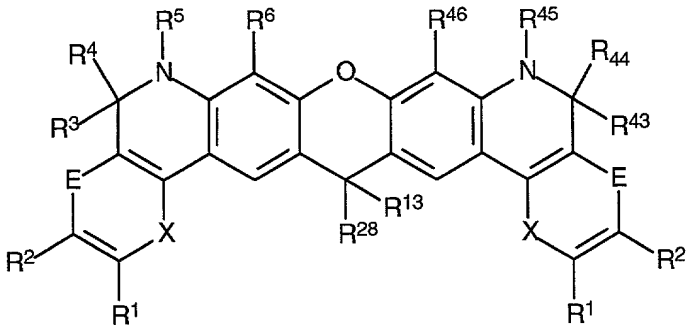
Selected dye embodiments are interconvertible with the reduced, or dihydroxanthene, form of the dye. These dihydroxanthene derivatives having the general structures:



or



or



where J´ is the same as J, defined above, R^{28} is defined as above, R^{13} is H, hydroxy, CN or a C_1-C_6 alkoxy, and R^{14} is H, C_1-C_{18} alkyl, or -L-R_x, or -L-S_c. The remaining dye substituents are as defined above.

In another embodiment of the reduced dyes of the invention, wherein R^{30} is a carboxylic acid, or R^{28} is a propionic or butyric acid, may exist in equilibrium with an isomer that incorporates a spirolactone ring. Similarly, reduced dyes wherein R^{30} is a sulfonic acid, or R^{28} is a sulfonic acid-substituted ethyl or propyl may exist in equilibrium with an isomer that incorporates a spirosultone ring. Isomers that incorporate a spirolactone or spirosultone ring are typically non-fluorescent until the ring is opened.

For these embodiments, R^{13} taken in combination with R^{28} forms a 5-membered spirolactone ring or a 5-membered spirosultone ring. Alternatively, R^{13} in combination with R^{30} forms a 5- or 6-membered spirolactone ring or a 5- or 6-membered spirosultone ring.

The dihydroxanthene and xanthylium versions of the dyes of the invention are freely interconvertible by well-known oxidation or reduction reagents, as discussed below.

Conjugates of Reactive Dyes

In one embodiment of the invention, the dye contains at least one group -L-R_x, where R_x is the reactive group that is attached to the fluorophore by a covalent linkage L (described below). The dyes with a reactive group (R_x) fluorescently label a wide variety of

organic or inorganic substances that contain or are modified to contain functional groups with suitable reactivity. resulting in chemical attachment of the conjugated substance (S_c), represented by -L-S_c. The reactive group and functional group are typically an electrophile and a nucleophile that can generate a covalent linkage. Alternatively, the reactive group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the reactive dye and the substance to be conjugated results in one or more atoms of the reactive group R_x to be incorporated into a new linkage L attaching the dye to the conjugated substance S_c. Selected examples of functional groups and linkages are shown in Table 2, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

Table 2: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones
aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers

alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers
boronates	glycols	boronate esters
carboxylic acids	amines/anilines	carboxamides
carboxylic acids	alcohols	esters
carboxylic acids	hydrazines	hydrazides
carbodiimides	carboxylic acids	N-acylureas or anhydrides
diazoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters
silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers

sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula -COΩ, where Ω is a good leaving group (e.g. succinimidyloxy ($-OC_4H_4O_2$) sulfosuccinimidyloxy ($-OC_4H_3O_2-SO_3H$), -1-oxybenzotriazolyl ($-OC_6H_4N_3$); or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-OCOR^a$ or $-OCNR^aNHR^b$, where R^a and R^b, which may be the same or different, are C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, or C_1-C_6 alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

** Acyl azides can also rearrange to isocyanates

The covalent linkage L binds the reactive group R_X or conjugated substance S_C to the compound, either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, carbon-sulfur bonds, phosphorus-oxygen bonds, and phosphorus-nitrogen bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. In one embodiment, the covalent linkage incorporates a platinum atom, such as described in U.S. Patent No. 5,714,327 (incorporated by reference). Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is a combination of single carbon-carbon bonds and carboxamide or thioether bonds. The longest linear segment of the linkage L preferably contains 4–10 nonhydrogen atoms, including one or two heteroatoms. Examples of L include substituted or unsubstituted polymethylene, arylene, alkylarylene, arylenealkyl, or arylthio. In one embodiment, L contains 1-6 carbon atoms; in another, L is a thioether linkage. In yet another embodiment, L is or incorporates the formula $-(CH_2)_a(CONH(CH_2)_b)_z-$, where a has any value from 0-5, b has any value from 1-5 and z is 0 or 1.

The -L-R_x and -L-S_c moieties are bound directly to the fluorophore at any of R^1, $R^{1'}$, R^2, $R^{2'}$, R^3, R^4, R^5, R^6, R^7, R^8, R^{15}, R^{16}, R^{20}, R^{21}, R^{23}, R^{24}, R^{25}, R^{30}, R^{31}, R^{32}, R^{33}, R^{34}, R^{37}, R^{38}, R^{41}, R^{42}, R^{43}, R^{44}, R^{45}, or R^{46}. In one embodiment, exactly one of R^{30}, R^{31}, R^{32}, R^{33} and R^{34} is an -L-R_x or -L-S_c moiety. In another embodiment, one of R^{37} and R^{38} is -L-R_x or -L-S_C. In yet another embodiment, R^{28} is -L-R_x or -L-S_C. In yet another embodiment, exactly one of R^{15} and R^{16} is an -L-R_x or -L-S_c moiety. In another embodiment, one of R^1 and R^2 is an -L-R_x and -L-S_C. In yet another embodiment, one of R^3 and R^4 is an -L-R_x or -L-S_C.

Choice of the reactive group used to attach the fluorophore to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the organic or inorganic substances include, but are not limited to, amines, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, sulfonate esters, purines, pyrimidines, carboxylic acids, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides), or a variety of sites may occur (e.g. amines, thiols, alcohols, phenols), as is typical for proteins. A conjugated substance may be conjugated to more than one fluorophore, which may be the same or different, or to a substance that is additionally modified by a hapten, such as biotin. Although some selectivity can be obtained by careful control of the reaction conditions, selectivity of labeling is best obtained by selection of an appropriate reactive dye.

Typically, R_X will react with an amine, a thiol, an alcohol, an aldehyde or a ketone. In one embodiment, R_X is an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, an amine, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine (including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, or a thiol group. By "reactive platinum complex" is meant chemically reactive platinum complexes such as described in U.S. Patent No. 5,714,327 (incorporated by reference).

Where the reactive group is a photoactivatable group, such as an azide, diazirinyl or azidoaryl derivative, the dye becomes chemically reactive only after illumination with light of an appropriate wavelength.

Where R_x is a succinimidyl ester of a carboxylic acid, the reactive dye is particularly useful for preparing dye-conjugates of proteins or oligonucleotides. Where R_x is a maleimide or haloacetamide the reactive dye is particularly useful for conjugation to thiol-containing substances. Where R_x is a hydrazide, the reactive dye is particularly useful for conjugation to periodate-oxidized carbohydrates and glycoproteins, and in addition is an aldehyde-fixable polar tracer for cell microinjection.

Preferably, R_X is a phosphoramidite, a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group, a perfluorobenzamido, or an azidoperfluorobenzamido group. More preferably, R_X is a phosphoramidite, a reactive platinum complex, or a succinimidyl ester of a carboxylic acid. Where R_X is a reactive platinum complex, it is typically a haloplatinate.

The reactive dyes of the invention are useful for the preparation of any conjugated substance that possess a suitable functional group for covalent attachment of the fluorophore. Examples of particularly useful dye-conjugates include, among others, conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, amino acids, peptides, proteins, nucleic acids, nucleic acid polymers, carbohydrates, lipids, ion-complexing moieties, and non-biological polymers. Alternatively, these are conjugates of cells, cellular systems, cellular fragments, or subcellular particles. Examples include, among others, virus particles, bacterial particles, virus components, biological cells (such as animal cells, plant cells, bacteria, yeast, or protists), or cellular components. Reactive dyes typically label reactive sites at the cell surface, in cell membranes, organelles, or cytoplasm. Preferably the conjugated substance is an amino acid, peptide, protein, tyramine (see Example 42), polysaccharide, ion-complexing moiety, nucleoside, nucleotide, oligonucleotide, nucleic acid, hapten, psoralen,

drug, hormone, lipid, lipid assembly, polymer, polymeric microparticle, biological cell or virus. In one embodiment, conjugates of biological polymers such as peptides, proteins, oligonucleotides, nucleic acid polymers are also labeled with a second fluorescent or non-fluorescent dye, including an additional dye of the present invention, to form an energy-transfer pair.

In one embodiment, the conjugated substance (S_c) is an amino acid (including those that are protected or are substituted by phosphates, carbohydrates, or C_1 to C_{22} carboxylic acids), or is a polymer of amino acids such as a peptide or protein. Preferred conjugates of peptides contain at least five amino acids, more preferably 5 to 36 amino acids. Preferred peptides include, but are not limited to, neuropeptides, cytokines, toxins, protease substrates, and protein kinase substrates. Preferred protein conjugates include enzymes, antibodies, lectins, glycoproteins, histones, albumins, lipoproteins, avidin, streptavidin, protein A, protein G, phycobiliproteins and other fluorescent proteins, hormones, toxins and growth factors. Typically, the conjugated protein is an antibody, an antibody fragment, avidin, streptavidin, a toxin, a lectin, a hormone, or a growth factor. Typically where the conjugated substance is a toxin, it is a neuropeptide or a phallotoxin, such as phalloidin.

In another embodiment, the conjugated substance (S_c) is a nucleic acid base, nucleoside, nucleotide or a nucleic acid polymer, including those that were modified to possess an additional linker or spacer for attachment of the dyes of the invention, such as an alkynyl linkage (US Pat. 5,047,519), an aminoallyl linkage (US Pat. 4,711,955) or other linkage. In another embodiment, the conjugated substance is a nucleoside or nucleotide analog that links a purine or pyrimidine base to a phosphate or polyphosphate moiety through a noncyclic spacer (acyclonucleosides and acyclonucleotides). Preferably, the conjugated nucleotide is a nucleoside triphosphate or a deoxynucleoside triphosphate or a dideoxynucleoside triphosphate.

Preferred nucleic acid polymer conjugates are labeled, single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates (AntiVirals, Inc.,

Corvallis OR), or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units. When the nucleic acid is a synthetic oligonucleotide, it typically contains fewer than 50 nucleotides, more typically fewer than 25 nucleotides.

Large fluorescent nucleic acid polymers are typically prepared from labeled nucleotides or oligonucleotides using oligonucleotide-primed DNA polymerization, such as by using the polymerase chain reaction or through primer extension, or by terminal-transferase catalyzed addition of a labeled nucleotide to a 3′-end of a nucleic acid polymer. Typically, the dye is attached via one or more purine or pyrimidine bases through an amide, ester, ether or thioether bond; or is attached to the phosphate or carbohydrate by a bond that is an ester, thioester, amide, ether or thioether. Alternatively, dye conjugate of the invention is simultaneously labeled with a hapten such as biotin or digoxigenin, or to an enzyme such as alkaline phosphatase, or to a protein such as an antibody. Nucleotide conjugates of the invention are readily incorporated by DNA polymerase and can be used for *in situ* hybridization (Example 62) and nucleic acid sequencing (e.g., US Pats. 5,332,666; 5,171,534; and 4,997,928; and WO Appl. 94/05688). In another aspect of the invention, the oligonucleotide incorporates an aliphatic amine, which is then conjugated to an amine-reactive dye of the invention. In yet another aspect of the invention, the purine bases of the oligonucleotide react with a reactive platinum complex bound to a dye of the invention, yielding a dye-conjugate (Example 65).

In one embodiment, the conjugated oligonucleotides of the invention are aptamers for a particular target molecule, such as a metabolite, dye, hapten, or protein. That is, the oligonucleotides have been selected to bind preferentially to the target molecule. Methods of preparing and screening aptamers for a given target molecule have been previously described and are known in the art (for example U.S. Patent No. 5,567,588 to Gold (1996), incorporated by reference).

In another embodiment, the conjugated substance (S_c) is a carbohydrate that is typically a polysaccharide, such as a dextran, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. Alternatively, the carbohydrate is a

polysaccharide that is a lipopolysaccharide. Preferred polysaccharide conjugates are dextran, FICOLL, or lipopolysaccharide conjugates.

In another embodiment, the conjugated substance (S_c), is a lipid (typically having 6–60 carbons), including glycolipids, phospholipids, sphingolipids, and steroids. Alternatively, the conjugated substance is a lipid assembly, such as a liposome. The lipophilic moiety may be used to retain the conjugated substances in cells, as described in US Pat. 5,208,148. Certain polar dyes of the invention may also be trapped within lipid assemblies.

Conjugates having an ion-complexing moiety serve as indicators for calcium, sodium, magnesium, zinc, potassium, or other biologically important metal ions. Preferred ion-complexing moieties are crown ethers, including diaryldiaza crown ethers (US Pat. 5,405,975); derivatives of 1,2-bis-(2-aminophenoxyethane)-*N,N,N',N'*-tetraacetic acid (BAPTA chelators; US Pat. 5,453,517, US Pat. 5,516,911, and US Pat. 5,049,673); derivatives of 2-carboxymethoxy-aniline-N,N-diacetic acid (APTRA chelators; AM. J. PHYSIOL. **256**, C540 (1989)); or pyridine- and phenanthroline-based metal ion chelators (U.S. Patent No. 5,648,270). Preferably the ion-complexing moiety is a diaryldiaza crown ether, a BAPTA chelator, or an APTRA chelator. Alternatively, where the dye is a derivative of a rhodol or a seminaphthorhodafluor (SNARF), the dye itself acts as an indicator of H^+ at pH values within about 1.5 pH units of the individual dye's pKa (see Example 44).

Where the conjugated substance of the -L-S_c moiety is an ion-complexing moiety, -L--S_c is typically R^{28}, or one of R^{30}-R^{34}. Alternatively, the ion-complexing moiety is bound at one of R^{37} or R^{38}. The ion indicators are optionally conjugated to plastic or biological polymers such as dextrans or microspheres to improve their utility as sensors.

Other conjugates of non-biological materials include dye-conjugates of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, or polymeric microparticles, including magnetic and non-magnetic microspheres, conducting and non-conducting metals and non-metals, and glass and plastic surfaces and

particles. Conjugates are optionally prepared by copolymerization of a dye that contains an appropriate functionality while preparing the polymer, or by chemical modification of a polymer that contains functional groups with suitable chemical reactivity. Other types of reactions that are useful for preparing dye-conjugates of polymers include catalyzed polymerizations or copolymerizations of alkenes and reactions of dienes with dienophiles, transesterifications or transaminations. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other structure.

The preparation of dye conjugates using reactive dyes is well documented, e.g. by R. Haugland, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Chapters 1-3 (1996); and Brinkley, BIOCONJUGATE CHEM., **3**, 2 (1992). Conjugates typically result from mixing appropriate reactive dyes and the substance to be conjugated in a suitable solvent in which both are soluble. The dyes of the invention are readily soluble in aqueous solutions, facilitating conjugation reactions with most biological materials. For those reactive dyes that are photoactivated, conjugation requires illumination of the reaction mixture to activate the reactive dye.

Labeled members of a specific binding pair are typically used as fluorescent probes for the complementary member of that specific binding pair, each specific binding pair member having an area on the surface or in a cavity that specifically binds to and is complementary with a particular spatial and polar organization of the other. Preferred specific binding pair members are proteins that bind non-covalently to low molecular weight ligands, such as biotin, drug-haptens and fluorescent dyes (such as an anti-fluorescein antibody). Such probes optionally contain a covalently bound moiety that is removed by an enzyme or light, or the dye is a dihydroxanthene derivative where R^{13} is H and the compound fluoresces following oxidation. Representative specific binding pairs are shown in Table 3.

Table 3. Representative Specific Binding Pairs

antigen	antibody

biotin	avidin (or streptavidin or anti-biotin)
IgG*	protein A or protein G
drug	drug receptor
toxin	toxin receptor
carbohydrate	lectin or carbohydrate receptor
peptide	peptide receptor
protein	protein receptor
enzyme substrate	enzyme
DNA (RNA)	aDNA (aRNA)†
hormone	hormone receptor
ion	chelator
psoralen	nucleic acid
target molecule	RNA or DNA aptamer

* IgG is an immunoglobulin

† aDNA and aRNA are the antisense (complementary) strands used for hybridization

In one aspect of the invention, the conjugated substance is further labeled with additional dye moieties, such that fluorescence energy is either accepted from, or transferred to, the dye of the invention. As stated above, N-aryl derivatives of the dyes of the invention have particular utility as quenchers in FRET applications. However, fluorescent dyes of the invention also possess utility as energy donors in FRET applications.

Applications and Methods of Use

In one aspect of the invention, the dye compounds of the invention possess utility as laser dyes according to methods known in the art. As discussed above, the long wavelength properties of the subject dyes allow the use of inexpensive laser diodes as excitation sources for dye lasers utilizing the subject dyes.

In another aspect of the invention, the dye compounds of the invention are used to directly stain or label a sample so that the sample can be identified or quantitated. For instance, such dyes may be added as part of an assay for a biological target analyte, as a detectable tracer element in a biological or non-biological fluid, or for such purposes as photodynamic therapy of tumors, in which a dyed sample is irradiated to selectively destroy tumor cells and tissues, or to photoablate arterial plaque or cells, usually through the photosensitized production of singlet oxygen.

In one aspect of the invention, the sample is obtained directly from a liquid source or as a wash from a solid material (organic or inorganic) or a growth medium in which cells have been introduced for culturing, or a buffer solution in which cells have been placed for evaluation. Where the sample comprises cells, the cells are optionally single cells, including microorganisms, or multiple cells associated with other cells in two or three dimensional layers, including multicellular organisms, embryos, tissues, biopsies, filaments, biofilms, etc.

Alternatively, the sample is a solid, optionally a smear or scrape or a retentate removed from a liquid or vapor by filtration. In one aspect of the invention, the sample is obtained from a biological fluid, including separated or unfiltered biological fluids such as urine, cerebrospinal fluid, blood, lymph fluids, tissue homogenate, interstitial fluid, cell extracts, mucus, saliva, sputum, stool, physiological secretions or other similar fluids. Alternatively, the sample is obtained from an environmental source such as soil, water, or air; or from an industrial source such as taken from a waste stream, a water source, a supply line, or a production lot.

In yet another embodiment, the sample is present on or in solid or semi-solid matrix. In one aspect of the invention, the matrix is a membrane. In another aspect, the matrix is an electrophoretic gel, such as is used for separating and characterizing nucleic acids or proteins. In another aspect, the matrix is a silicon chip or glass slide, and the analyte of interest has been immobilized on the chip or slide in an array. In yet another aspect, the matrix is a microwell plate or microfluidic chip, and the sample is analyzed by automated

methods, typically by various methods of high-throughput screening, such as drug screening.

The dye compounds of the invention are generally utilized by combining a dye compound of the invention as described above with the sample of interest under conditions selected to yield a detectable optical response. The term "dye compound" is used herein to refer to all aspects of the claimed dyes, including both reactive and non-reactive dyes and conjugates of dyes. The dye compound typically forms a covalent or non-covalent association or complex with an element of the sample, or is simply present within the bounds of the sample or portion of the sample. The sample is then illuminated at a wavelength selected to elicit the optical response. Typically, staining the sample is used to determine a specified characteristic of the sample by further comparing the optical response with a standard or expected response.

A detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or instrumentally. Typically the detectable response is a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. The degree and/or location of staining, compared with a standard or expected response, indicates whether and to what degree the sample possesses a given characteristic. Some dyes of the invention, such as N-aryl rhodamines and triphenyl methane derivatives, may exhibit little fluorescence emission, but are still useful as chromophoric dyes. Such chromophores are useful as energy acceptors in FRET applications, or to simply impart the desired color to a sample or portion of a sample.

For biological applications, the dye compounds of the invention are typically used in an aqueous, mostly aqueous or aqueous-miscible solution prepared according to methods generally known in the art. The exact concentration of dye compound is dependent upon the experimental conditions and the desired results, but typically ranges from about one nanomolar to one millimolar or more. The optimal concentration is determined by systematic variation until satisfactory results with minimal background fluorescence is

accomplished.

The dye compounds are most advantageously used to stain samples with biological components. The sample may comprise heterogeneous mixtures of components (including intact cells, cell extracts, bacteria, viruses, organelles, and mixtures thereof), or a single component or homogeneous group of components (e.g. natural or synthetic amino acid, nucleic acid or carbohydrate polymers, or lipid membrane complexes). These dyes are generally non-toxic to living cells and other biological components, within the concentrations of use.

The dye compound is combined with the sample in any way that facilitates contact between the dye compound and the sample components of interest. Typically, the dye compound or a solution containing the dye compound is simply added to the sample. Certain dyes of the invention, particularly those that are substituted by one or more sulfonic acid moieties, tend to be impermeant to membranes of biological cells, and once inside viable cells are typically well retained. Treatments that permeabilize the plasma membrane, such as electroporation, shock treatments or high extracellular ATP can be used to introduce dye compounds into cells. Alternatively, the dye compounds can be physically inserted into cells, e.g. by pressure microinjection, scrape loading, patch clamp methods, or phagocytosis.

Dyes that incorporate an amine or a hydrazine residue can be microinjected into cells, where they can be fixed in place by aldehyde fixatives such as formaldehyde or glutaraldehyde. This fixability makes such dyes useful for intracellular applications such as neuronal tracing.

Dye compounds that possess a lipophilic substituent, such as phospholipids, will non-covalently incorporate into lipid assemblies, e.g. for use as probes for membrane structure; or for incorporation in liposomes, lipoproteins, films, plastics, lipophilic microspheres or similar materials; or for tracing. Lipophilic dyes are useful as fluorescent probes of membrane structure.

Chemically reactive dye compounds will covalently attach to a corresponding functional group on a wide variety of materials, forming dye conjugates as described above. Using dye compounds to label reactive sites on the surface of cells, in cell membranes or in intracellular compartments such as organelles, or in the cell's cytoplasm, permits the determination of their presence or quantity, accessibility, or their spatial and temporal distribution in the sample. Photoreactive dyes can be used similarly to photolabel components of the outer membrane of biological cells or as photo-fixable polar tracers for cells.

Optionally, the sample is washed after staining to remove residual, excess or unbound dye compound. The sample is optionally combined with one or more other solutions in the course of staining, including wash solutions, permeabilization and/or fixation solutions, and solutions containing additional detection reagents. An additional detection reagent typically produces a detectable response due to the presence of a specific cell component, intracellular substance, or cellular condition, according to methods generally known in the art. Where the additional detection reagent has, or yields a product with, spectral properties that differ from those of the subject dye compounds, multi-color applications are possible. This is particularly useful where the additional detection reagent is a dye or dye-conjugate of the present invention having spectral properties that are detectably distinct from those of the staining dye.

The compounds of the invention that are dye conjugates are used according to methods extensively known in the art; e.g. use of antibody conjugates in microscopy and immunofluorescent assays; and nucleotide or oligonucleotide conjugates for nucleic acid hybridization assays and nucleic acid sequencing (e.g., US Patent Nos. 5,332,666 to Prober, et al. (1994); 5,171,534 to Smith, et al. (1992); 4,997,928 to Hobbs (1991); and WO Appl. 94/05688 to Menchen, et al.; all incorporated by reference). Dye-conjugates of multiple independent dyes of the invention possess utility for multi-color applications.

At any time after or during staining, the sample is illuminated with a wavelength of

light selected to give a detectable optical response, and observed with a means for detecting the optical response. Equipment that is useful for illuminating the dye compounds of the invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optionally integrated into laser scanners, fluorescence microplate readers, standard or minifluorometers, or chromatographic detectors.

The optical response is optionally detected by visual inspection, or by use of any of the following devices: CCD cameras, video cameras, photographic film, laser-scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes. Where the sample is examined using a flow cytometer, examination of the sample optionally includes sorting portions of the sample according to their fluorescence response.

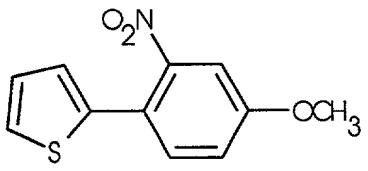
Kits

One aspect of the instant invention is the formulation of kits that facilitate the practice of various assays using the dyes of the invention, as described above. The kits of the invention typically comprise a colored or fluorescent dye of the invention, either present as a chemically reactive label useful for preparing dye-conjugates, or present as a dye-conjugate where the conjugated substance is a specific binding pair member, or a nucleoside, nucleotide, oligonucleotide, nucleic acid polymer, peptide, or protein. The kit optionally further comprises one or more buffering agents, typically present as an aqueous solution. The kits of the invention optionally further comprise additional detection reagents, a purification medium for purifying the resulting labeled substance, luminescence standards, enzymes, enzyme inhibitors, organic solvent, or instructions for carrying out an assay of the invention.

The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of this invention.

Example 1. Preparation of Compound 1.

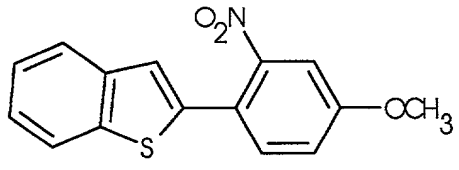
The following compound is prepared:



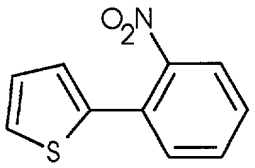
Compound 1

To a flask (250 mL) is added 4-bromo-3-nitroanisole (57.7 mmol), thiophene-2-boronic acid (69.2 mmol), $Pd(OAc)_2$ (4.5 mmol), K_2CO_3 (14.3 mmol) and Bu_4NBr (115 mmol). The flask is flushed with N_2 gas and equipped with a rubber septum. To the reaction mixture is added deoxygenated water (120 mL) with a syringe, and the suspension is stirred and degassed to remove residual oxygen. The suspension is stirred and heated for 1 h at 80 °C under N_2 gas, then cooled to room temperature and diluted with water. The resulting precipitate is filtered and washed with ethyl acetate. The filtrate is extracted with ethyl acetate, and the combined organic layers are dried over anhydrous Na_2SO_4 and volatiles are removed under vacuum to give a brown solid. The crude solid is purified on a silica gel column eluting with hexanes and 5:1 hexanes/ethyl acetate to give Compound 1 (15.2 g).

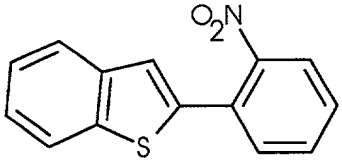
Compounds 2, 3, and 4 are prepared analogously, using benzothiophene-2-boronic acid and 4-bromo-3-nitroanisole, thiophene-2-boronic acid and 2-bromonitrobenzene, and benzothiophene-2-boronic acid and 2-bromonitrobenzene, respectively.



Compound 2



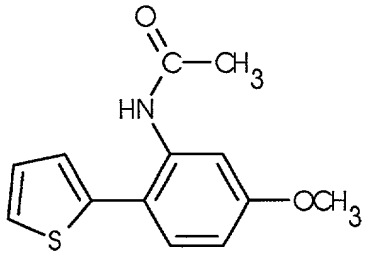
Compound 3



Compound 4

Example 2. Preparation of Compound 5.

The following compound is prepared:



Compound 5

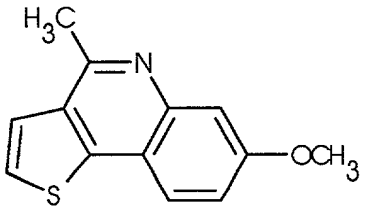
Compound 1 (75 mmol) is dissolved in 1:1 THF/methanol (160 mL). To the solution is added Zn dust (1.3 moles), followed by the dropwise addition of concentrate hydrochloric acid until analysis by thin layer chromatography (TLC) showed that Compound 1 and the reduction intermediates are completely consumed. Excess Zn is removed by filtration and washed with THF. The combined filtrates are concentrated *in vacuo*, and the residue is poured into water (1 L), and neutralized to pH = 7-8 with 2 M NaOH. The resulting suspension is extracted with ethyl acetate. The organic phase is washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give the crude amino derivative.

The amino derivative is dissolved in THF (200 mL), and to the resulting solution is added Ac_2O (20 mL) and pyridine (20 mL) at room temperature. The reaction mixture is stirred at room temperature for 5-6 h, and then heated at reflux for 1-2 h. The mixture is

concentrated to an oil, which is then redissolved in ethyl acetate (500 mL) and washed with 5% HCl and brine respectively. The ethyl acetate solution is dried over anhydrous Na_2SO_4, concentrated and loaded on a silica gel column, which is eluted with a gradient of hexanes/ethyl acetate to give Compound 5 (16.2 g).

Example 3. Preparation of Compound 6.

The following compound is prepared:

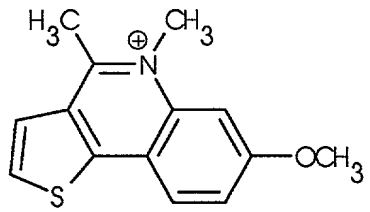


Compound 6

Compound 5 (65.6 mmol) is put in a dry flask (150 mL). To the flask is carefully added $POCl_3$ (80 mL). The mixture is stirred and heated at 80 °C for 30 min. The solution is concentrated *in vacuo*, and the residue is diluted with chloroform (500 mL). The chloroform solution is washed with ice/water (250 mL) and 5% ammonia (250 mL), then dried over anhydrous Na_2SO_4, and evaporated to afford a brown crude product. The crude product is further purified on a silica gel column using chloroform as the eluant to yield Compound 6 (10.2 g).

Example 4. Preparation of Compound 7.

The following compound is prepared:

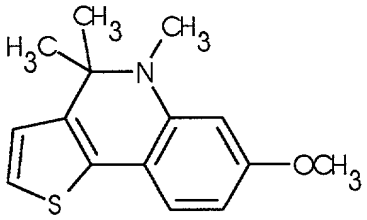


Compound 7

To a solution of Compound 6 (44.5 mmol) in chlorobenzene (150 mL), is added *p*-TsOMe (237 mmol), and the resulting mixture is heated at reflux for 2 days. The reaction mixture is then cooled to room temperature, and the resulting precipitate is collected by filtration. The resulting crude product is purified by recrystallization from ethyl acetate to give Compound 7 (10.5 g).

Example 5. Preparation of Compound 8.

The following compound is prepared:

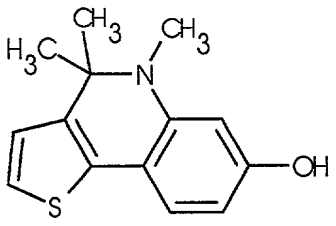


Compound 8

A solution is prepared of Compound 7 (7.2 mmol) in dry THF (50 mL) at 0 °C under N_2 gas. To the solution is slowly added MeMgCl (30 mmol) in THF. The reaction mixture is slowly warmed to room temperature and stirred for 2 days. The suspension is carefully poured onto crushed ice and acidified to pH = 2 with 2 M HCl. The solution is then extracted with ethyl acetate, washed with brine and dried over anhydrous Na_2SO_4. The solution is concentrated *in vacuo* and purified on a silica gel column using 5:1 hexanes/ethyl acetate as the eluant to give Compound 8 (600 mg).

Example 6. Preparation of Compound 9.

The following compound is prepared:

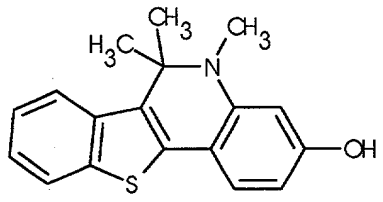


Compound 9

To a solution of Compound 8 (1.25 mmol) in dry CH_2Cl_2 (20 mL) is added BBr_3 (0.6 mmol) at 0 °C, and the mixture is stirred at room temperature for 1 h. The reaction mixture is concentrated *in vacuo*, and cooled in a dry ice/acetone bath. To the residue is slowly added methanol (10 mL) to quench the residual BBr_3, followed by the addition of 100 mL water. The solution is neutralized to pH = 7-8 with saturated $NaHCO_3$, extracted with ethyl acetate and washed with brine. The ethyl acetate solution is dried over anhydrous Na_2SO_4 and concentrated. The resulting residue is purified on a silica gel column using a gradient of hexanes/ethyl acetate as eluant to give Compound 9 (280 mg).

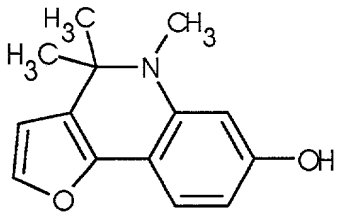
Example 7. Preparation of Compounds 10-12.

The following compounds are prepared:



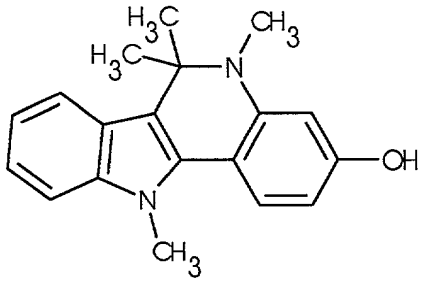
Compound 10

Compound 10 is prepared from Compound 2 following the procedures of Examples 2-6.



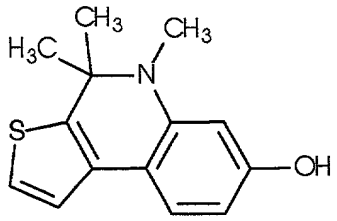
Compound 11

Compound 11 is prepared from furan-2-boronic acid following the procedures of Examples 1-6.



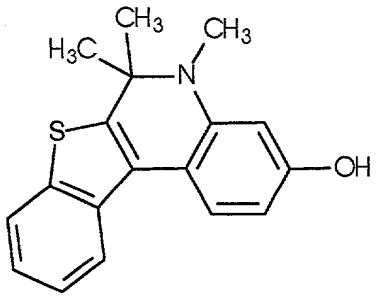
Compound 12

Compound 12 is prepared from N-methylindole-2-boronic acid following the procedures of Examples 1-6.



Compound 60

Compound 60 is prepared from thiophene-3-boronic acid following the procedures of Examples 1-6.

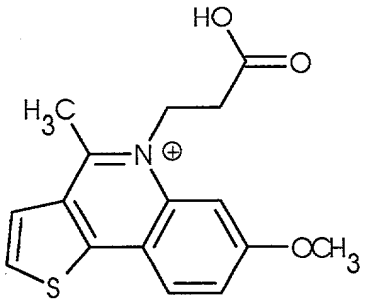


Compound 61

Compound 61 is prepared from benzothiophene-3-boronic acid following the procedures of Examples 1-6.

Example 8. Preparation of Compound 13.

The following compound is prepared:

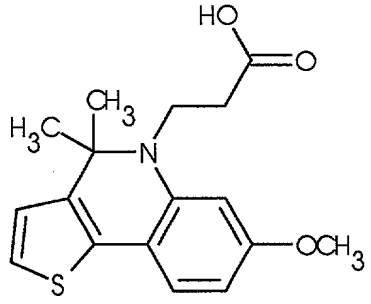


Compound 13

To a solution of Compound 6 (10 mmol) in chlorobenzene (50 mL), is added β-propiolactone (20 mmol). The solution is heated at reflux for 2 days, then cooled to room temperature, and the resulting precipitate collected by filtration. The crude product is purified by recrystallization from ethyl acetate to give Compound 13 (1.2 g).

Example 9. Preparation of Compound 14.

The following compound is prepared:

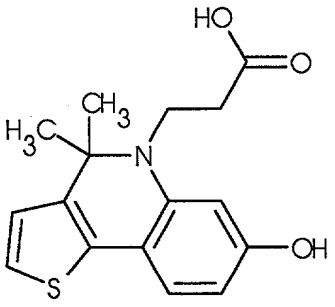


Compound 14

Compound 13 is treated with MeMgCl to give Compound 14, following the procedure of Example 5.

Example 10. Preparation of Compound 15.

The following compound is prepared:

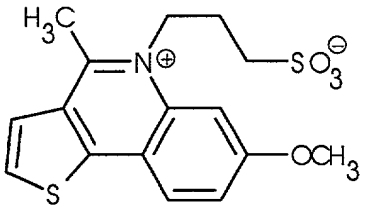


Compound 15

Compound 14 is treated with BBr_3 to give Compound 15, following the procedure of Example 6.

Example 11. Preparation of Compound 16.

The following compound is prepared:

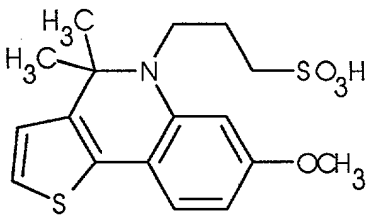


Compound 16

To a solution of Compound 6 (20 mmol) in chlorobenzene (80 mL), is added 1,3-propanesultone (30 mmol), and the reaction mixture is heated at reflux for 3 days. After cooling to room temperature, the reaction mixture is filtered and the precipitate collected. The crude material is washed with chloroform and recrystallized from isopropyl alcohol to give Compound 16 (1.4 g).

Example 12. Preparation of Compound 17.

The following compound is prepared:

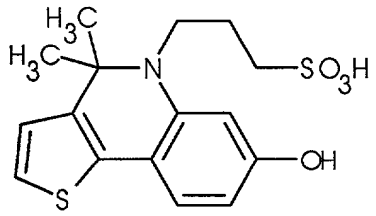


Compound 17

Compound 16 is treated with MeMgCl to give Compound 17, following the procedure of Example 5.

Example 13. Preparation of Compound 18.

The following compound is prepared:

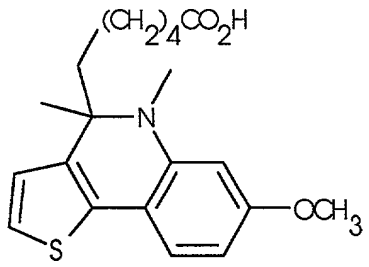


Compound 18

Compound 16 is treated with BBr_3 to give Compound 18, following the procedure of Example 6.

Example 14. Preparation of Compound 19.

The following compound is prepared:

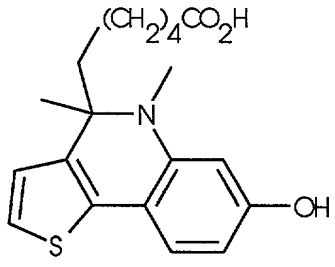


Compound 19

To a solution of Compound 7 (6 mmol) in dry THF (100 mL) is slowly added 6-(2,4,10-trioxatricyclo [3.3.1.1] dec-3-yl)hexylmagnesium bromide (50 mmol) in THF at 0 °C under N_2 gas. The reaction mixture is slowly warmed to room temperature, and stirred for 2 days. The suspension is carefully poured onto crushed ice, and acidified to pH = 2 with 2 M HCl. The solution is extracted with ethyl acetate, and evaporated *in vacuo* to dryness. The residue is redissolved in methanol (25 mL), 5 M HCl (10 mmol) is added, and the solution is stirred at room temperature overnight. The reaction mixture is concentrated *in vacuo*, poured into water, and extracted with ethyl acetate. The combined organic layers are washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give a crude product, which is further purified by chromatography on silica gel using 4:1 chloroform/methanol as the eluant to give Compound 19 (615 mg).

Example 15. Preparation of Compound 20.

The following compound is prepared:

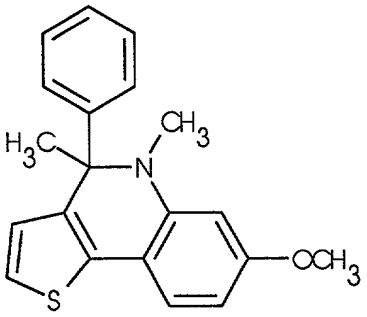


Compound 20

Compound 19 is treated with BBr_3 to give Compound 20, following the procedure of Example 6.

Example 16. Preparation of Compound 21.

The following compound is prepared:

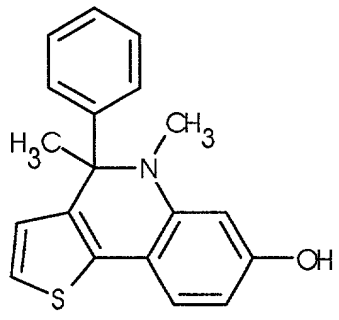


Compound 21

To a solution of Compound 7 (7.2 mmol) in dry THF (50 mL) is slowly added PhMgCl (30 mmol) in THF at 0 °C under N_2 gas. The reaction mixture is slowly warmed to room temperature, and stirred for 2 days. The resulting suspension is carefully poured onto crushed ice, acidified to pH = 2 with 2 M HCl, extracted with ethyl acetate, washed with brine and dried over anhydrous Na_2SO_4. The solution is then concentrated *in vacuo* and the residue is purified by chromatography on silica gel using 5:1 hexanes/ethyl acetate as eluant to give Compound 21 (656 mg).

Example 17. Preparation of Compound 22.

The following compound is prepared:



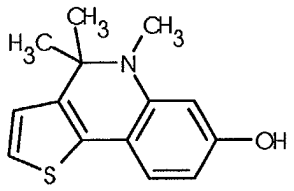
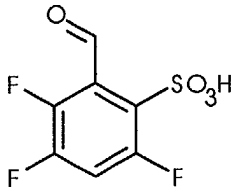
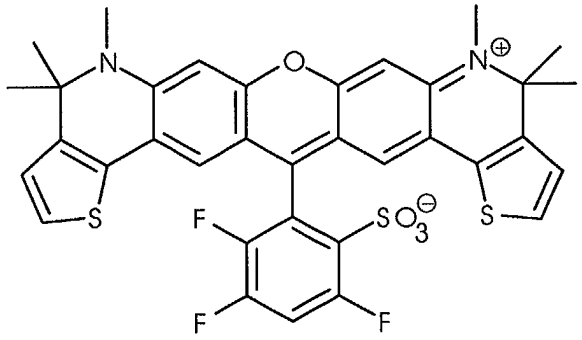
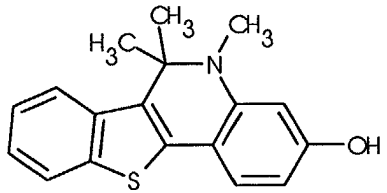
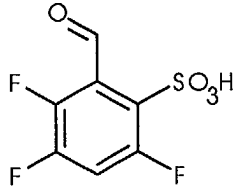
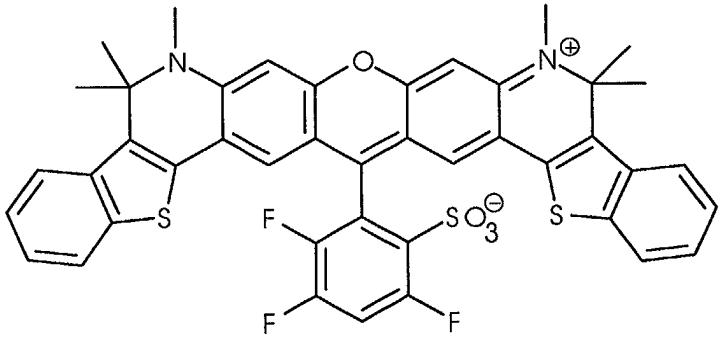
Compound 22

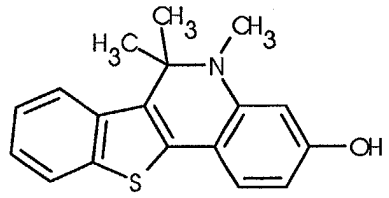
Compound 21 is treated with BBr_3 to give Compound 22, following the procedure of Example 6.

Example 18. Preparation of Compounds 23-36.

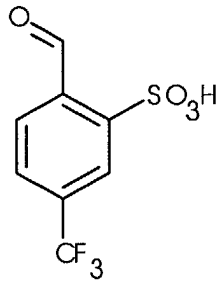
The following general method is utilized to prepare Compounds 23-36. A derivatized 3-aminophenol (2 mmol) and a 2-sulfobenzaldehyde precursor (1 mmol) are dissolved in DMF. To the DMF solution is slowly added trifluoroacetic acid (TFA, 1 ml) at room temperature. The reaction mixture is stirred overnight, then heated to 110-120 °C for 7-10 h. The mixture is concentrated *in vacuo*, the residue is dissolved in a minimal amount of chloroform, and purified by chromatography on silica gel to yield the desired product.

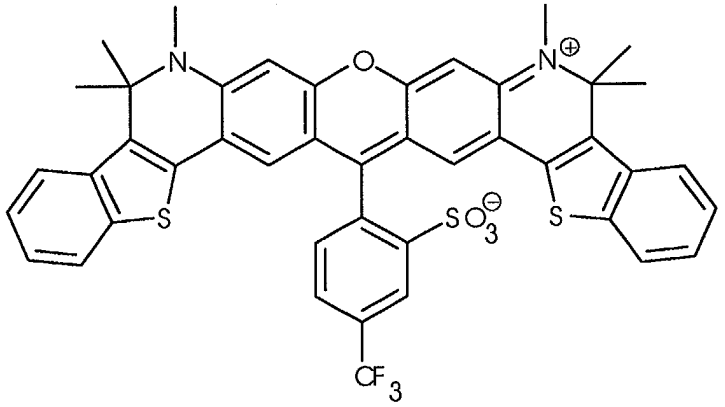
Table 4

3-aminophenol precursor	2-sulfobenzaldehyde precursor	Product
 Compound 9		 Compound 23
 Compound 10		 Compound 24

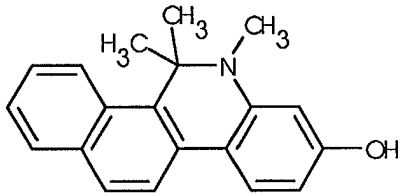


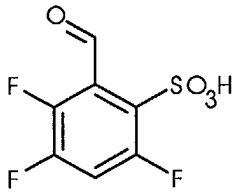
Compound 10

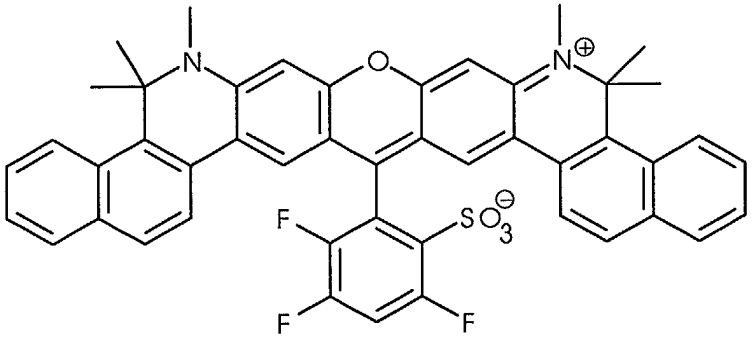




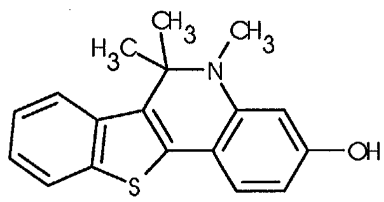
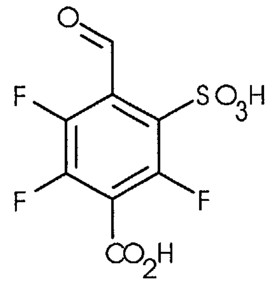
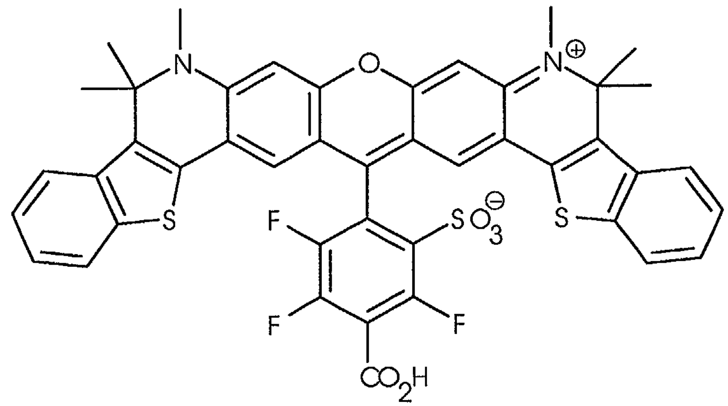
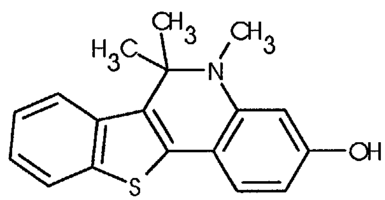
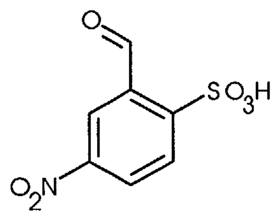
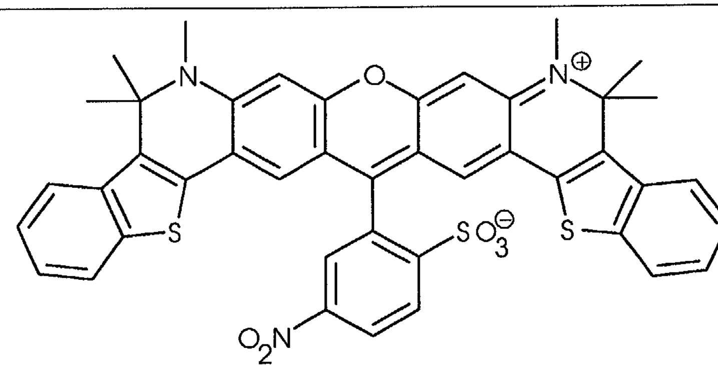
Compound 25

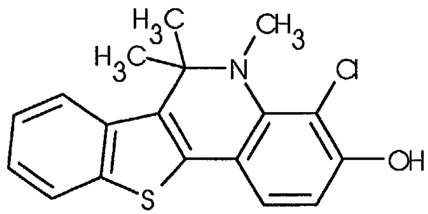
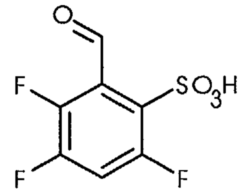
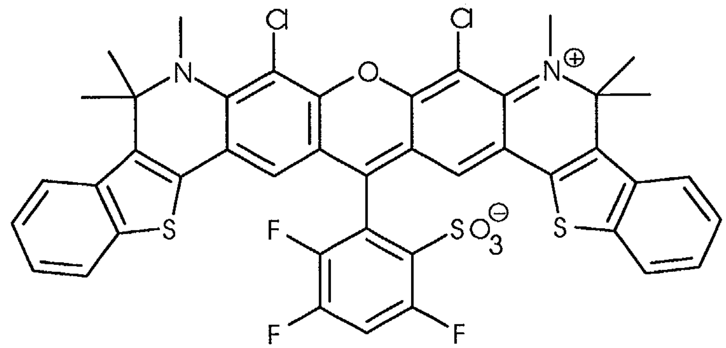
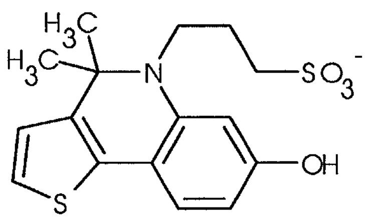
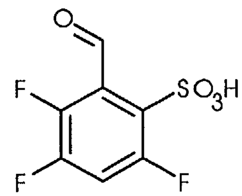
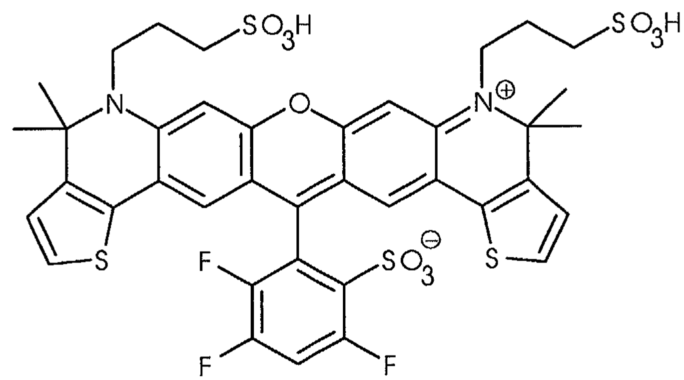


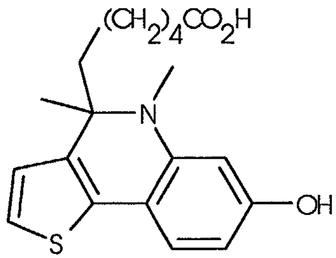
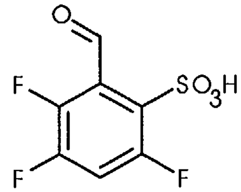
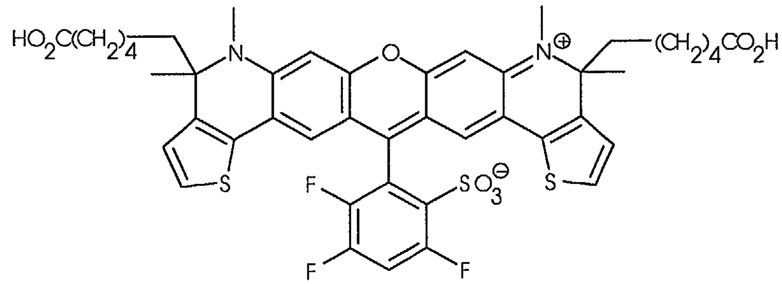
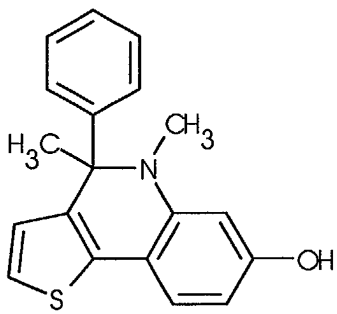
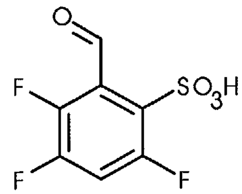
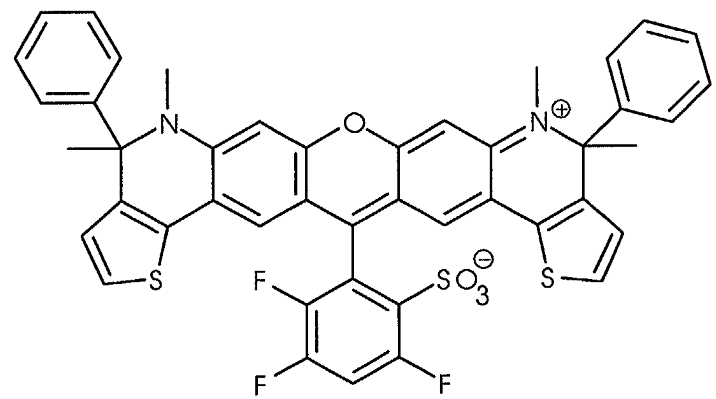


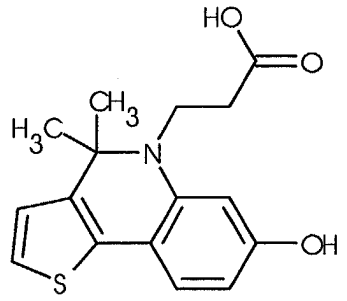


Compound 26

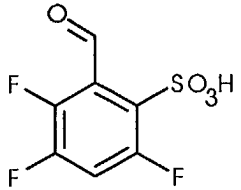
 Compound 10		 Compound 27
 Compound 10		 Compound 28

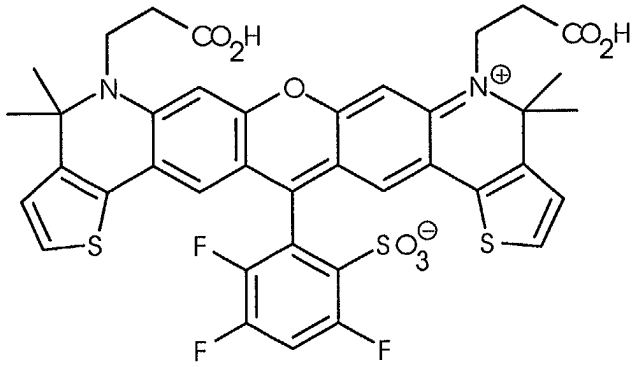
		 Compound 29
 Compound 18		 Compound 30

 Compound 20		 Compound 31
 Compound 22		 Compound 32

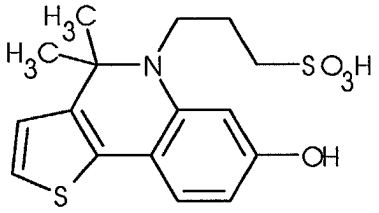


Compound 15

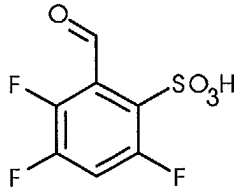


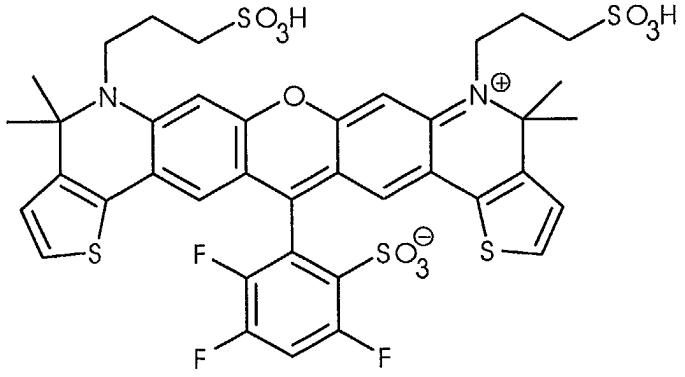


Compound 33

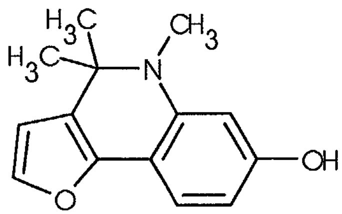
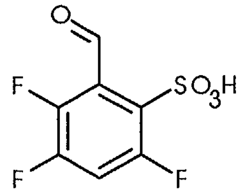
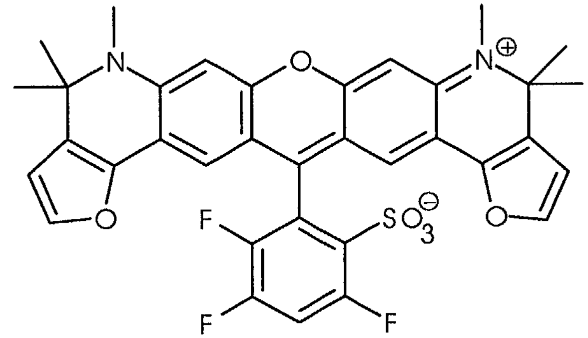
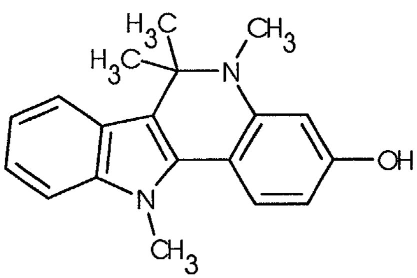
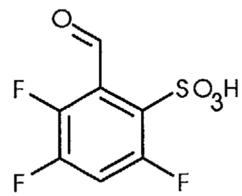
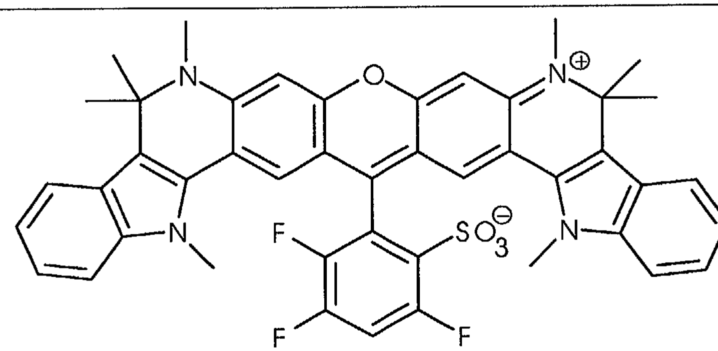


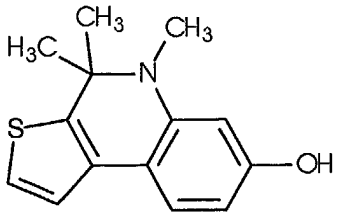
Compound 18



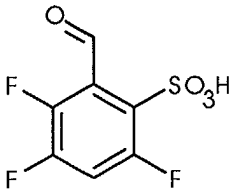


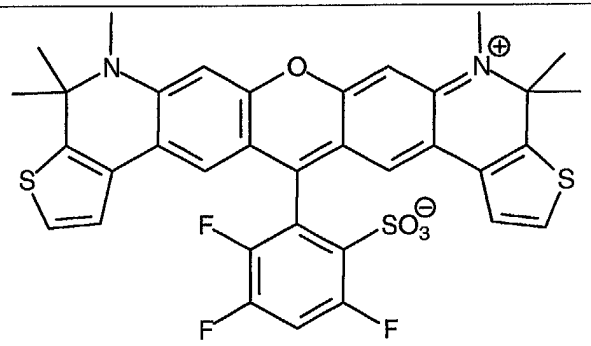
Compound 34

 Compound 11		 Compound 35
 Compound 12		 Compound 36



Compound 60

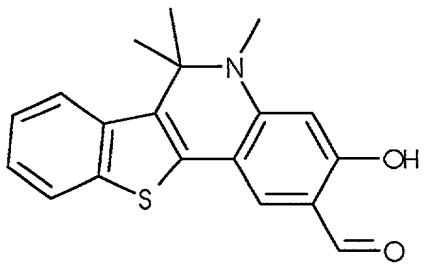




Compound 62

Example 19. Preparation of Compound 37.

The following compound is prepared:

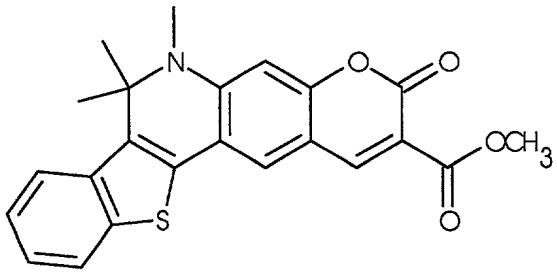


Compound 37

$POCl_3$ (0.3 mL) is dissolved in dry DMF (8 mL) at 0 °C, and the mixture is stirred at room temperature for 0.5 h. To the reaction mixture is added Compound 10 (2 mmol) in DMF (2 mL). The resulting solution is carefully poured into cold 10% $NaHCO_3$, and extracted with ethyl acetate. The combined organic layers are washed with brine and dried over anhydrous Na_2SO_4. The solution is evaporated *in vacuo* to give Compound 37 (516 mg).

Example 20. Preparation of Compound 38.

The following compound is prepared:



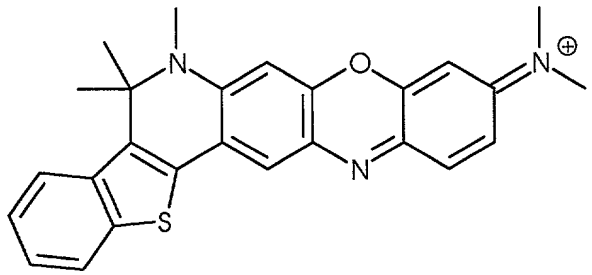
Compound 38

To a solution of Compound 37 (2 mmol) and dimethylmalonate (2.5 mmol) in methanol (10 mL) is added piperidine (0.1 mL). The reaction mixture is heated at reflux for 7 h, and concentrated *in vacuo*. The resulting residue is poured into water, and extracted

with ethyl acetate. The combined organic layers are washed with brine and dried over anhydrous Na_2SO_4. The solution is evaporated under vacuum, and the resulting crude material is purified by chromatography on silica gel using 50:1 chloroform/methanol as the eluant to give Compound 38 (256 mg).

Example 21. Preparation of Compound 39.

The following compound is prepared:

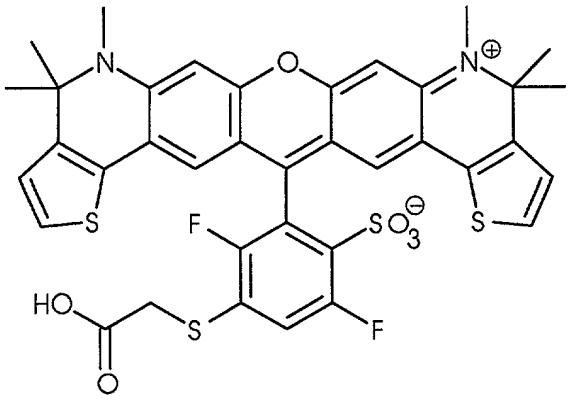


Compound 39

A solution of Compound 10 (1 mmol) and N,N-dimethyl-3-hydroxy-4-nitrosoaniline (1.6 mmol) in 1:1 butanol/toluene (10 mL) is heated at reflux for 16 h with a Dean-Stark trap. The reaction mixture is concentrated *in vacuo*, and the residue is poured into water and extracted with ethyl acetate. The combined organic layers are washed with brine and dried over anhydrous Na_2SO_4. The organic solution is evaporated under vacuum, and the resulting crude material is purified by chromatography on silica gel eluting with 5:1 chloroform/methanol to give Compound 39 (56 mg).

Example 22. Preparation of Compound 40.

The following compound is prepared:



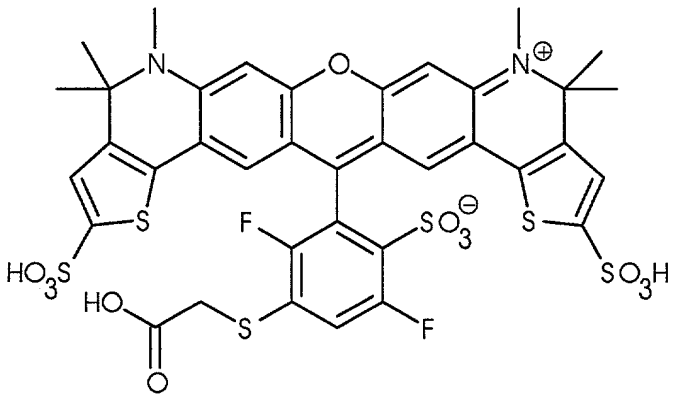
Compound 40

To a solution of Compound 23 (0. 14 mmol) in dimethylacetamide (60 mL) is added thioacetic acid (1.5 mL) at room temperature. The reaction mixture is heated at 110-120 °C for 2 h, then cooled to room temperature and poured into water. The suspension is extracted with chloroform, washed with water, and the chloroform extract is dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue is purified on a silica gel column using 20:1, 10:1, 5:1 chloroform/methanol and 3:1: 0.01 chloroform/methanol/acetic acid as the eluants to give Compound 40 (90 mg).

Compound 62 (Example 18) is treated with thioacetic acid according to the above procedure to yield the analogous 6-carboxymethylthio derivative, Compound 63.

Example 23. Preparation of Compound 41.

The following compound is prepared:



Compound 41

To a solution of Compound 40 (0.12 mmol) in 98% sulfuric acid (5 mL) is slowly added 30% fuming sulfuric acid (4 mL) at 0 °C. The reaction mixture is stirred for 10 min at 0 °C, and poured into cold ether. The resulting precipitate is collected, washed with ether, and redissolved in methanol. The methanol solution is neutralized to pH = 7-8 with saturated Li_2CO_3, and the resulting white precipitate (Li_2SO_4) is removed by filtration and washed with methanol. The filtrate is concentrated and purified by HPLC to give Compound 41 (60 mg).

The fluorescence quantum yield of Compound 41 is determined using sulforhodamine 101 (in ethyl alcohol) as the reference standard (Φ_F = 0.90). The concentrations of sulforhodamine 101 (the reference) and Compound 41 are adjusted to obtain an absorbance of 0.25 (in 1 cm cell) at 560 nm. The sample and reference are each excited at 560 nm and their fluorescence spectra obtained. The fluorescence quantum yield of the sample (Φ_F^X) in the indicated solvent was calculated from the following formula, considering that the peak area (A_R) of the emission spectrum of the reference and that of the tested dye (A_X) can be readily determined:

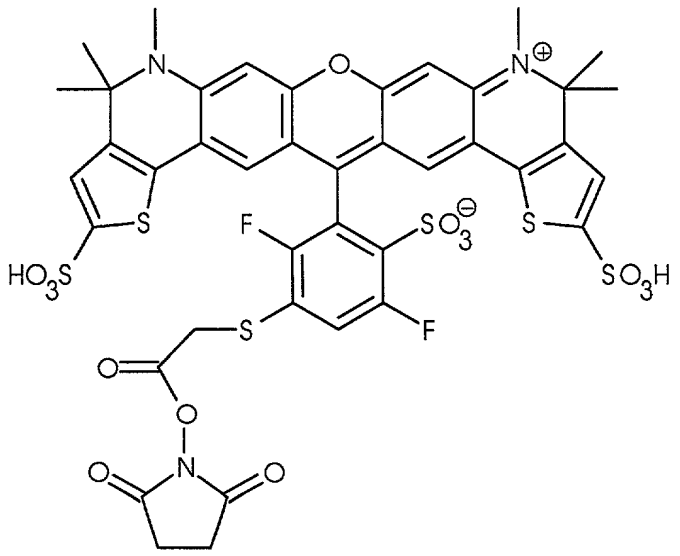
$$\Phi_F^X = A_x \Phi_F^R / A_R$$

where Φ_F^R and Φ_F^X are the fluorescence quantum yields of the reference and the testing dye. The measurements are done in triplicate and the estimated errors are no more than 1%. The quantum yields of Compound 41 are determined to be 0.72 (in methyl alcohol) and 0.68 (in water) respectively.

Compound 63 is sulfonated using the above procedure to yield the analogous disulfonate derivative, Compound 64.

Example 24. Preparation of Compound 42.

The following compound is prepared:

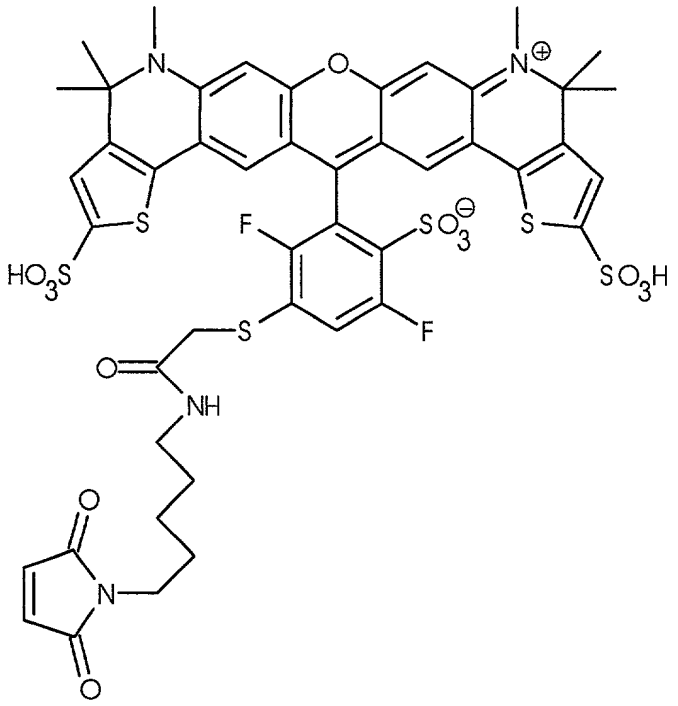


Compound 42

To a solution of Compound 41 (0.019 mmol) in dry DMF (10 mL) is added *N,N'*-disuccinimidylcarbonate (0.23 mmol) and 4-dimethylaminopyridine (0.04 mmol) at room temperature, and the mixture is stirred for 6 h. The solution is then concentrated *in vacuo*, and the residue is suspended in ethyl acetate (100 mL). The precipitate is collected and washed with ethyl acetate. The crude solid is redissolved in dry DMF, ethyl acetate (100 mL) is added, and the resulting precipitate is collected, washed with ethyl acetate, and dried to give Compound 42 (20 mg).

Example 25. Preparation of Compound 43.

The following compound is prepared:

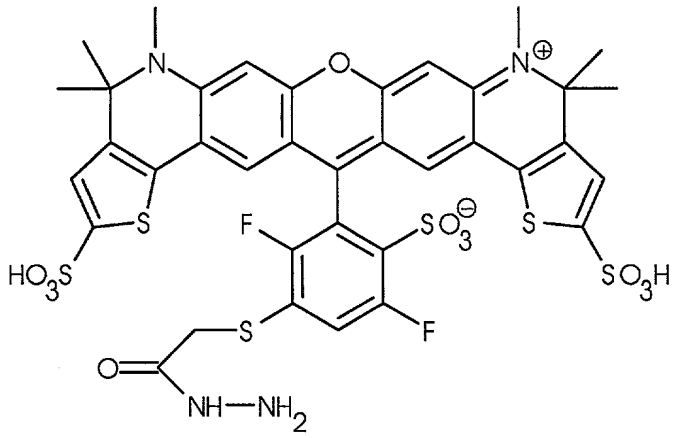


Compound 43

To a solution of Compound 42 (0.1 mmol) in anhydrous DMF (2 mL) is slowly added 1 mL DMF solution of N-(5-aminopentyl)maleimide (0.12 mmol). To the reaction mixture is then added triethylamine (0.15 mmol), and the resulting mixture is stirred at room temperature for 5-8 h. The solution is then concentrated under vacuum and poured into ethyl acetate. The resulting precipitate is collected by filtration, and washed with ethyl acetate. The crude product is purified by HPLC to give Compound 43 (26 mg).

Example 26. Preparation of Compound 44.

The following compound is prepared:

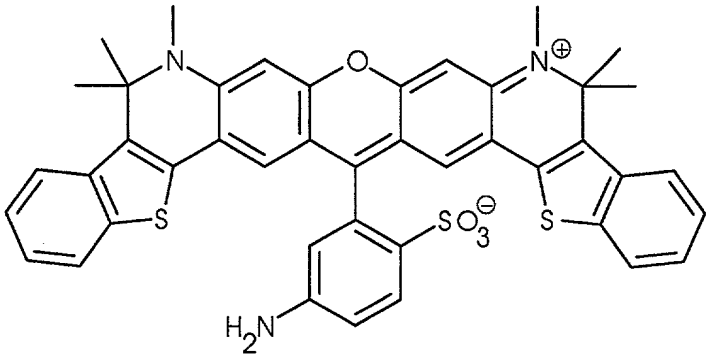


Compound 44

To a solution of hydrazine (0.5 mmol) in DMF (0.05 mL) is slowly added 2 mL DMF solution of Compound 42 (0.1 mmol). The resulting mixture is stirred at room temperature for 5-8 h. The reaction solution is then concentrated under vacuum and poured into isopropyl alcohol. The resulting precipitate is collected by filtration, and washed with isopropyl alcohol. The crude material is purified by HPLC to give Compound 44 (39 mg).

Example 27. Preparation of Compound 45.

The following compound is prepared:



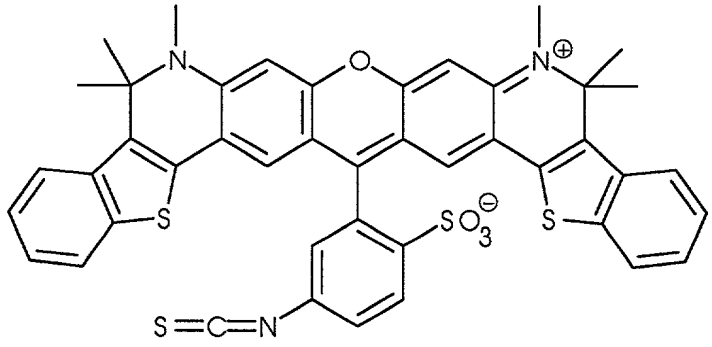
Compound 45

Compound 28 (1 mmol) is dissolved in 10:1 methanol/water (60 mL). To the resulting solution is added Na_2S (1.5 moles) and NaSH (4.5 mmol). The reaction mixture is heated at reflux for 1.5 h, then concentrated *in vacuo*. The residue is poured into water

(250 mL) and neutralized to pH =7-8 with 2 M HCl. The resulting suspension is extracted with chloroform, the organic phase is washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The resulting crude product is further purified by chromatography on silica gel, eluting with a gradient of chloroform/methanol to give Compound 45.

Example 28. Preparation of Compound 46.

The following compound is prepared:

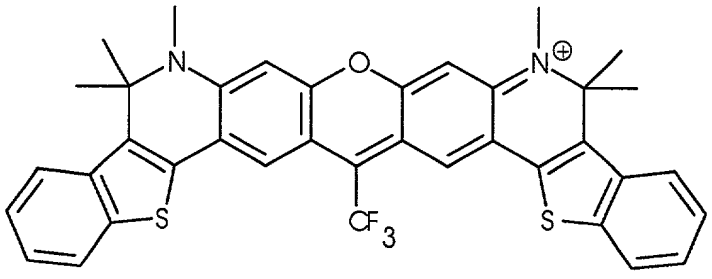


Compound 46

Compound 45 (0.1 mmol) is dissolved in dry DMF (10 mL) and thiophosgene (1 mmol) is added. The reaction mixture is stirred at room temperature for 6 h, concentrated *in vacuo*, and poured into ether. The resulting precipitate is collected, and washed with ether to give Compound 46 (45 mg).

Example 29. Preparation of Compound 47.

The following compound is prepared:

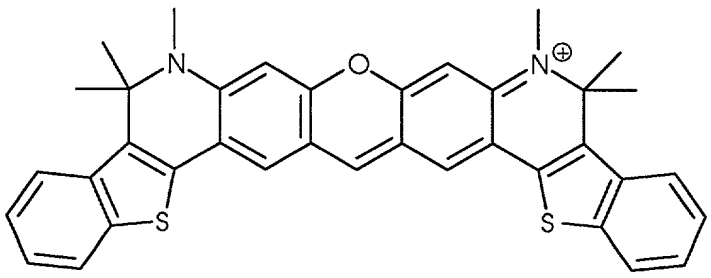


Compound 47

Compound 10 (1 mmol), trifluoroacetic anhydride (5 mmol) and TFA (1.5 mmol) are dissolved in CH_2Cl_2 (2.5 mL). The reaction mixture is stirred for 2 days, then concentrated *in vacuo*, and redissolved in ethanol (1 mL) containing 70% perchloric acid (0.1 g). The precipitate is collected, and purified on a silica gel column using 10:1 chloroform/methanol to afford Compound 47 (128 mg).

Example 30. Preparation of Compound 48.

The following compound is prepared:

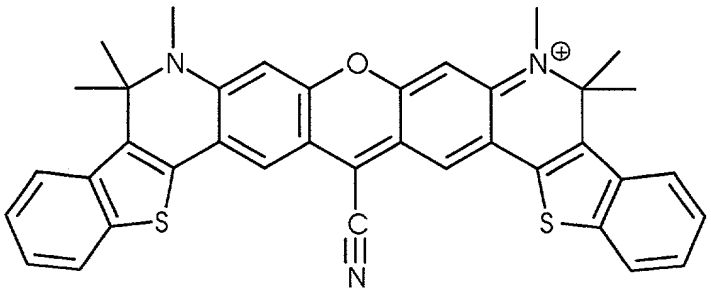


Compound 48

To a solution of Compound 10 (5 mmol) in ethanol (20 mL) is added 37% formaldehyde (20 mmol) at room temperature, and the mixture is stirred at room temperature for 2 days. The resulting yellow solid is collected and dried. To the solution of the crude solid in concentrated sulfuric acid (5 mL) is slowly added 1 M $NaNO_2$ at 0 °C. The solution is allowed to stand at room temperature for 2 days, and the precipitate is collected. The crude solid is purified by chromatography on silica gel using a gradient of chloroform/methanol to give Compound 48 (28 mg).

Example 31. Preparation of Compound 49.

The following compound is prepared:

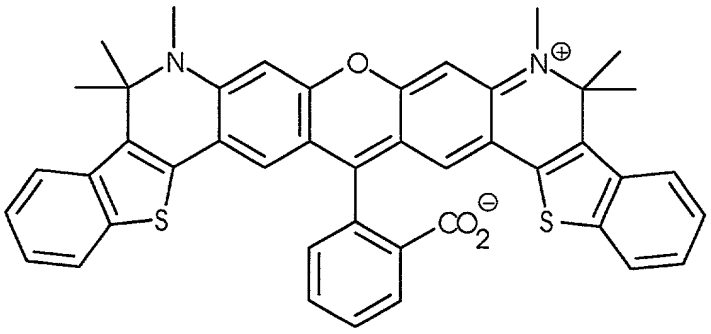


Compound 49

To a solution of Compound 48 (0.5 mmol) in DMF (20 mL) is added KCN (20 mmol) at room temperature, and the mixture is stirred at room temperature for 2 days. Oxygen gas is bubbled through the solution for 6 h. The solution is concentrated *in vacuo*, and purified by chromatography on silica gel, eluting with a gradient of chloroform/methanol to give Compound 49 (31 mg).

Example 32. Preparation of Compound 50.

The following compound is prepared:

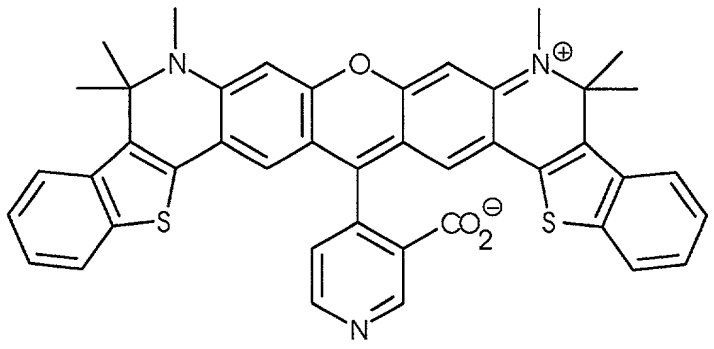


Compound 50

To a suspension of Compound 10 (1 mmol) and phthalic anhydride (0.4 mmol) in propionic acid (10 mL) is slowly added 98% sulfuric acid (0.1 mL). The reaction mixture is heated at 130-140 °C for 6 h, then carefully poured onto crushed ice, and extracted with ethyl acetate. The combined organic layers are washed with brine and dried over anhydrous Na_2SO_4. The solution is concentrated *in vacuo* and purified by chromatography on silica gelusing 5:1 chloroform/methanol as the eluant to give Compound 50 (356 mg).

Example 33. Preparation of Compound 51.

The following compound is prepared:

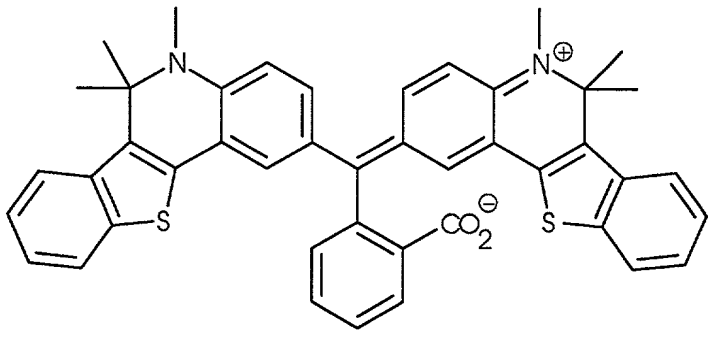


Compound 51

The reaction of Compound 10 (1 mmol) with compound pyridine-3,4-dicarboxylic anhydride (0.4 mmol) in propionic acid (10 mL) produces Compound 51 analogously with the procedure used to prepare Compound 50.

Example 34. Preparation of Compound 53.

The following compound is prepared:



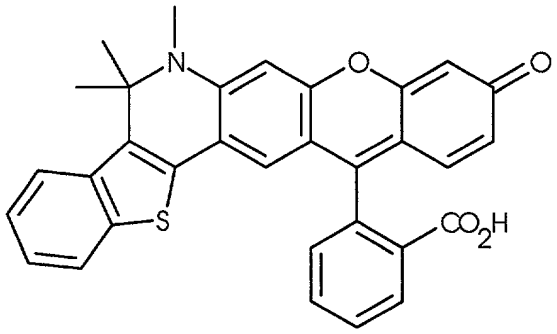
Compound 53

N-methyl-2,2-dimethyl-1,2-dihydrothianaphtheno [3,4] quinoline (Compound 52) is prepared from Compound 4 using the procedures of Examples 2-5. Compound 52 (1 mmol) and phthalic anhydride (0.45) are mixed well, and melted for 15 minutes *in vacuo*. The mixture is then cooled to room temperature, and extracted with methanol (50 mL). The

methanol solution is evaporated under vacuum, and the resulting crude solid is purified by chromatography on silica gel eluting with 10:1:1 chloroform/methanol/ethyl acetate to give Compound 53 (156 mg).

Example 35. Preparation of Compound 54.

The following compound is prepared:

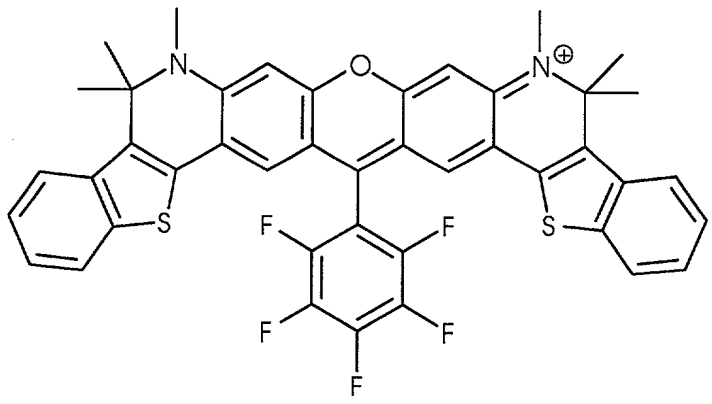


Compound 54

To a solution of Compound 10 (1 mmol) in methanesulfonic acid (5 mL) is slowly added 2-(2,4-dihydroxybenzoyl)benzoic acid (1.1 mmol). The reaction mixture is heated at 50-60 °C for 2 h. The solution is then carefully poured onto crushed ice, and extracted with ethyl acetate. The combined organic layers are washed with brine and dried over anhydrous Na_2SO_4. The solution is concentrated *in vacuo* and purified by chromatography on silica gel using 5:1 chloroform/methanol as the eluant to give Compound 54 (96 mg).

Example 36. Preparation of Compound 55.

The following compound is prepared:

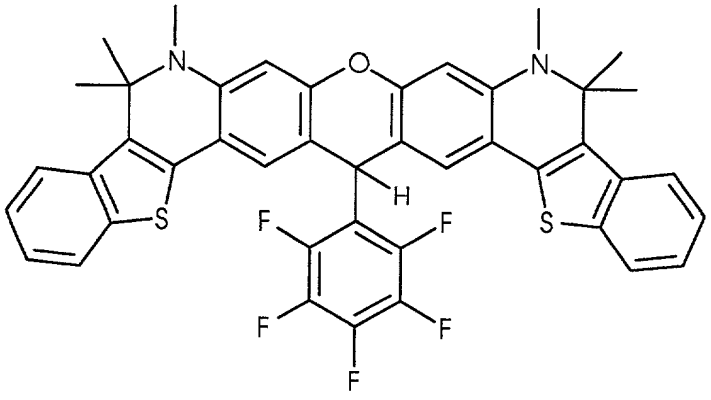


Compound 55

To a solution of Compound 10 (1 mmol) and pentafluorobenzaldehyde (0.4 mmol) in propionic acid (10 mL) is slowly added *p*-TsOH (25 mg). The reaction mixture is heated at 130-140 °C for 6 h, cooled to room temperature, and poured into water. The resulting solution is extracted with ethyl acetate, and the combined organic layers are washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The resulting crude material is purified by chromatography on silica gel eluting with 10:1 chloroform/methanol to give Compound 55 (156 mg).

Example 37. Preparation of Compound 56.

The following compound is prepared:



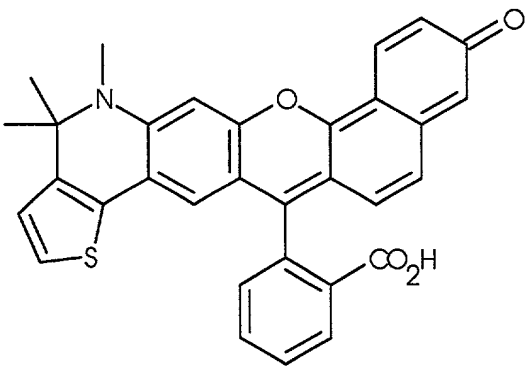
Compound 56

To a solution of Compound 55 (0.1 mmol) and in ethanol (5 mL) is slowly added $NaBH_4$ (5 mmol) at 0 °C. The reaction mixture is warmed to room temperature, stirred for

2 h, and poured into ice/water. The resulting suspension is extracted with ethyl acetate, and the combined organic layers are washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The resulting crude material is purified by chromatography on silica gel using 10:1 chloroform/ethyl acetate as the eluant to give Compound 56 (35 mg).

Example 38. Preparation of Compound 57.

The following compound is prepared:

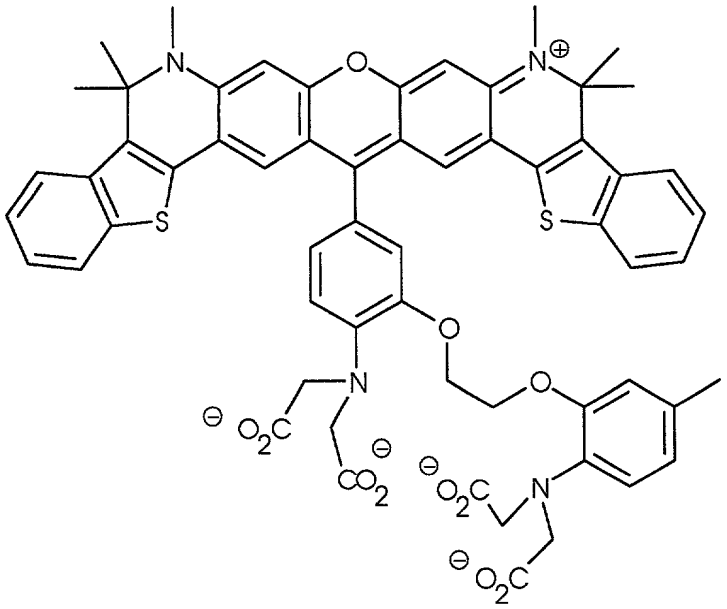


Compound 57

To a solution of 2-(2′-carboxybenzoyl)-1,6-dihydroxynaphthalene (1 mmol) in methanesulfonic acid (5 mL) is slowly added Compound 9 (1.1 mmol). The reaction mixture is heated at 50-60 °C for 2 h, the solution is carefully poured onto crushed ice, and extracted with ethyl acetate. The combined organic layers are washed with brine, dried over anhydrous Na_2SO_4, and concentrated under vacuum. The resulting residue is purified by chromatography on silica gel using 5:1 chloroform/methanol as eluant to give Compound 57 (76 mg).

Example 39. Preparation of Compound 58

The following compound is prepared:



Compound 58

Compound 10 (0.15 g, 0.51 mmol), 1-(2-bis(methoxycarbonylmethyl)amino-5-methyl)-2-(2-bis(methoxycarbonylmethyl)amino-5-formylphenoxy)ethane (0.146 g, 0.25 mmol), and catalytic p-TsOH are suspended in propionic acid (10 mL) under nitrogen atmosphere and sparged with nitrogen for 40 minutes while being heated to 60 °C. The reaction mixture is heated overnight. After cooling, the solution is poured into a solution of 11 g sodium acetate in 100 mL water with stirring. The resulting precipitate is collected by suction filtration, rinsed with water, and dried *in vacuo* to give 0.28 g of a pale green powder.

The intermediate (0.28 g; 0.25 mmol) is dissolved in methanol/chloroform (1:1, 12 mL) and treated with *p*-chloranil (0.123 g, 0.50 mmol). The resulting mixture is stirred at room temperature until TLC analysis shows consumption of starting material (approximately 1.5 hours). The reaction mixture is suction filtered and the filtrate concentrated *in vacuo*. The resulting dark blue residue is purified by flash chromatography on 35 g siliga gel using chloroform:methanol:acetic acid (50:5:1) as eluant. Pure product fractions are combined and concentrated *in vacuo* to give 52 mg of a dark blue solid.

All of the blue intermediate is converted to the free carboxylic acid form by treatment of 52 mg (40 mmol) with 1 M KOH (0.35 mL, 0.35 mmol) in dioxane/methanol

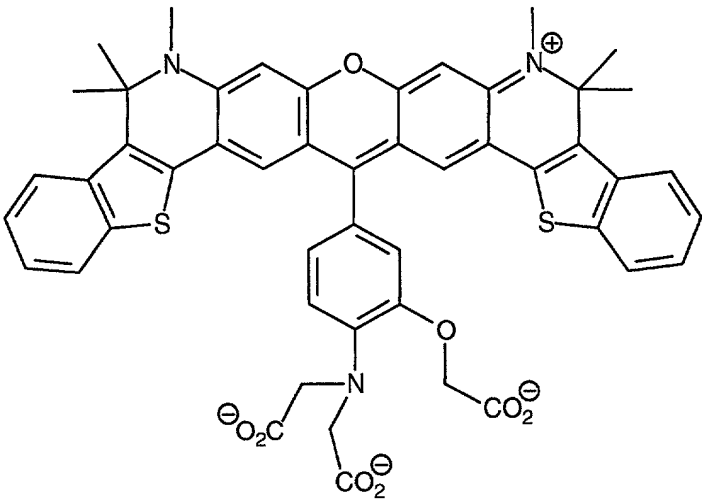
(5:3 mL). The resulting dark amber mixture is stirred at room temperature overnight, then concentrated *in vacuo*. The residue is suspended in 5 mL water, and the pH (13) is adjusted to 2 by the addition of aqueous HCl. The resulting blue precipitate is collected by filtration, rinsed with water, and dried under vacuum to yield 45 mg of a dark blue powder. The product is further purified dissolution in dilute aqueous KOH solution, followed by chromatography on SEPHADEX LH-20 resin using water as eluant. Pure product fractions are combined and lyophilized to give Compound 58 as a dark blue powder, $R_f = 0.45$ (dioxane:isopropyl alcohol:water:ammonium hydroxide 15:58:13:13)).

Example 40. Determination of the Ca^{2+} binding affinity of Compound 58.

The fluorescence response and dissociation constant of Compound 58 is determined using the method described by Tsien et al. METH. ENZYM. **172**, 230 (1989). Compound 58 (1 mg) is dissolved in deionized water and 5 µL of this solution is diluted into 3 mL of each of two buffers, which are cross diluted to arrive at a series of Ca^{2+} concentrations between zero and 35 µM. Emission spectra of the dye solutions are scanned between dilutions to generate a family of curves. Each of these curves has maximal fluorescence emission at approximately 640 nm with an increase in fluorescence emission intensity with increasing Ca^{2+} concentration. This intensity change is plotted against the concentration of free Ca^{2+} to give a value for the dissociation constant of the indicator. The calculated dissociation constant at 20 °C is 57.5 µM.

Example 41. Preparation of Compound 59.

The following compound is prepared:

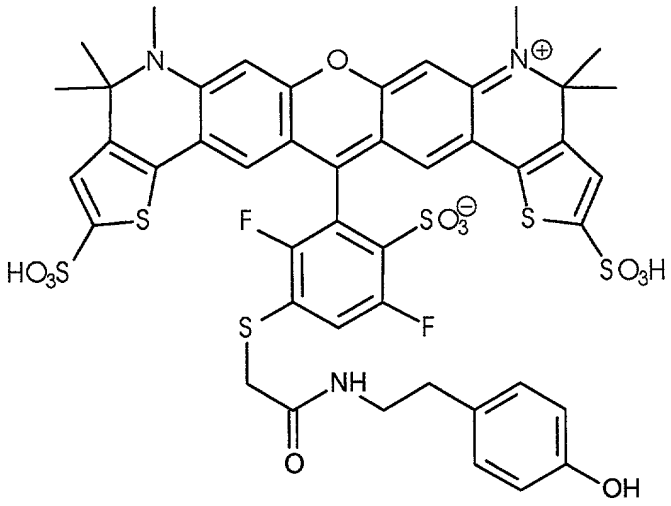


Compound 59

A light green mixture of 2-carboxymethoxy-4-formyl-aniline-N,N-diacetic acid, trimethyl ester (4-formyl APTRA, trimethyl ester; 60 mg, 0.17 mmol), Compound 10 (0.10 g, 0.34 mmol) and catalytic p-TsOH in 8 mL propionic acid is degassed *in vacuo*, flushed with nitrogen gas three times, then heated to 75 degrees with stirring in darkness overnight. After cooling, the reaction solution is poured into a solution of 10 g sodium acetate in 100 mL water. The resulting precipitate is collected by suction filtration, rinsed with water, and dried *in vacuo* to give 0.15 g of a green powder (100%, R_f 0.30 (EtOAc/hexanes)). All of this powder (0.17 mmol) is dissolved in 1:1 methanol:chloroform (10 mL), and p-chloranil (50 mg, 0.20 mmol) is added. The resulting mixture is stirred for 3 hours, then concentrated *in vacuo*. The residue is purified by preparative silica gel TLC, using chloroform:methanol:acetic acid (50:5:1) as eluant. The desired product band (R_f 0.20) is scraped from the plate. The product is extracted from the silica gel using chloroform:methanol:acetic acid (50:5:1), followed by filtration and concentration *in vacuo* to give 49 mg (31%) of a dark blue powder. This powder (40 mg, 0.041 mmol) is dissolved in 4 mL methanol and 1M KOH solution (0.26 mL, 0.26 mmol) is added. After stirring overnight, TLC shows product formation (R_f 0.15 (dioxane:isopropyl alcohol:water:ammonium hydroxide 15:58:13:13)) and starting material consumption (R_f 0.75). The reaction solution is added to 30 mL aqueous citric acid (pH 2). The resulting precipitate is collected on a Hirsch funnel, rinsed with water, and dried *in vacuo* to give Compound 59 as 25 mg of a blue powder.

Example 42. Preparation of a Tyramine Conjugate.

The following compound is prepared:



To a solution of Compound 42 (0.1 mmol) (Example 25) in anhydrous DMF (2 mL) is slowly added 1 mL DMF solution of tyramine (0.22 mmol). The resulted mixture is stirred at room temperature for 5-8 h until the dye is completely consumed. The reaction solution is concentrated *in vacuo*, and poured into ethyl acetate. The resulting precipitate is collected by filtration and washed with ethyl acetate. The crude material is further purified by HPLC to give the desired product.

Example 43. Labeling of Mitochondria in Live Cells.

The NIH/3T3 mouse fibroblast cell line is obtained from American Type Culture Collection Co., Rockville, Md. The cells are maintained in a humidified atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % calf serum, 50 µg/mL gentamicin, 300 µg/mL L-glutamine and 10 mM HEPES pH 7.4. Cells are subcultured every 3 days by trypsinization using 0.05 % trypsin and 0.02 % EDTA in a Ca- and Mg-free saline solution (Gibco BRL, Gaithersburg, MD). Cell passage number ranges from 120-122. To obtain well-spread single cells, 5 x 10^4 cells are plated onto 18 x 18 mm coverslips in 60 mm culture dishes.

A cationic dye, such as Compound 48, 49 or 55, is dissolved in DMSO/ethanol (1/1) to prepare a 5 mM stock solution. The stock solutions are kept sealed in an amber reagent bottle and stored at 4 °C. Each labeling medium is prepared by adding stock solution to fresh culture medium in an amount sufficient to make final dye concentrations of between 50 and 200 nM.

The 3T3 cells are transferred to the labeling medium containing the selected dye and incubated at 37 °C for 15 to 30 minutes. The cells are then washed with fresh medium and observed using a Zeiss Axioplan microscope equipped with a filter optimized for tetramethylrhodamine. The selected dyes stain mitochondria selectively and fluorescently.

Example 44. pH titration of Compound 57.

Compound 57 is first dissolved in a series of buffers that have each been calibrated using a pH meter. Acetate buffers are typically used in the range of pH 4–6, and phosphate buffers in the pH range 6–8. Absorption measurements are made using solutions that are approximately 10 μM in concentration, and fluorescence measurements are made using solutions that are approximately 1 μM in concentration. The absorption or emission data is then plotted versus pH to determine pKa values. For example, Figure 3 shows the fluorescence emission data for Compound 57 plotted versus the pH of the solution, when excited at 338 nm.

Example 45. Preparation of a phalloidin dye-conjugate.

To aminophalloidin *p*-toluenesulfonate (3.5 mg, 4 μmol) and Compound 42 (6.0 mg, 5 μmol) in DMF is added N,N-diisopropylethylamine (2 μL, 11 μmol). The mixture is stirred at room temperature for 3 hours. To this is added 7 mL of diethyl ether. The solid is collected by centrifugation. The crude product is purified on SEPHADEX LH-20, eluting with water to give the pure phalloidin conjugate.

Example 46. Preparation of a drug dye-conjugate.

A fluorescent dopamine D_2 antagonist is prepared as follows: To 10 mg of *N*-(*p*-aminophenethyl)spiperone (Amlaiky et al., FEBS LETT **176**, 436 (1984)), and 10 μL *N*,*N*-diisopropylethylamine in 1 mL of DMF is added 15 mg of Compound 42. After 3 hours, the reaction mixture is poured into 5 mL ether. The precipitate is centrifuged, then purified by chromatography on silica gel using 10–30% methanol in chloroform.

Example 47. Preparation of Protein dye-conjugates.

A series of dye conjugates of goat anti-mouse IgG, streptavidin and other proteins, including R-phycoerythrin (R-PE) are prepared by standard means (Haugland et al., METH. MOL. BIOL. **45**, 205 (1995); Haugland, METH. MOL. BIOL. **45**, 223 (1995); Haugland, METH. MOL. BIOL. **45**, 235 (1995)) using Compound 42 and a succinimidyl ester derivative of CY-5 dye.

A solution of the desired protein is prepared at 10 mg/mL in 0.1 M sodium bicarbonate. The labeling reagents are dissolved in DMF at 10 mg/mL. Predetermined amounts of the labeling reagents are added to the protein solutions with stirring. A molar ratio of 10 equivalents of dye to 1 equivalent of protein is typical, though the optimal amount varies with the particular labeling reagent, the protein being labeled and the protein's concentration, and is determined empirically. The reaction mixture is incubated at room temperature for one hour, or on ice for several hours. The dye–protein conjugate is typically separated from free unreacted reagent by size-exclusion chromatography on BIO-RAD P-30 resin equilibrated with PBS. The initial, protein-containing colored band is collected and the degree of substitution is determined from the absorbance at the absorbance maximum of each fluorophore, using the extinction coefficient of the free fluorophore.

Table X: Fluorescence of Selected Protein Conjugates of the Invention

Protein	DOS	Quantum Yield
Goat anti-Mouse IgG	1.2	1.14

Streptavidin	3.60	0.98
Wheat Germ Agglutinin	1.13	0.56
Conconavilin A	1.10	0.43
Goat anti-Rabbit IgG (highly absorbed)	1.50	1.36
Goat anti-Chicken IgG	1.40	0.99
Rabbit anti-Mouse IgG	1.30	1.10
Goat anti-Mouse IgG (highly absorbed)	1.30	1.40
Goat anti-Guinea Pig IgG	1.20	1.40
Protein A (MR=4)	2.20	1.44
Protein A (MR=8)	4.40	0.68
Transferrin (MR=20)	1.30	1.20

*Extinction coefficients are determined for the free carboxylic acid in aqueous solution

Protein conjugates of antibody fragments, of other avidins and of other proteins are prepared and analyzed similarly.

Example 48. Fluorescent labeling of periodate-oxidized proteins.

Two samples of 5 mg each of goat IgG antibody in 1 mL of 0.1 M acetate, 0.135 M NaCl, pH 5.5 are treated with 2.1 mg of sodium metaperiodate on ice, for 1 and 2 hours, respectively. The reactions are stopped by addition of 30 µL ethylene glycol. The antibodies are purified on a MATREX GH 25 column (1 cm × 30 cm) packed in PBS pH 7.2. One-tenth volume of 1 M sodium bicarbonate is added to increase the pH and Compound 44 is added at a molar ratio of dye to protein of 50:1. The reaction is stirred for 2 hours at room temperature. Sodium cyanoborohydride is added to a final concentration of 10 mM and the reaction is stirred for 4 hours at room temperature. The antibody conjugates are

purified by dialysis and on MATREX GH 25 columns as described above. Antibodies that are oxidized for 1 hour typically yield a degree of substitution of 1 mole of dye per mole of IgG. Antibodies that are oxidized for 2 hours typically yield a degree of substitution of approximately 2 mole of dye per mole of IgG.

Example 49. Total fluorescence of selected dye–protein conjugates as a function of degree of substitution.

A series of goat anti-mouse IgG conjugates is prepared as in Example 47 so as to yield derivatives with similar degrees of substitution (DOS). The fluorescence emission spectra of a goat anti-mouse IgG conjugate of Compound 42 (DOS = 1.2) and a goat anti-mouse IgG conjugate of CY-5 dye (DOS 2.5) at the same solution optical densities, excited at 600 nm, and in comparison to a solution of DDAO dye having the same absorbance used as a fluorescence standard. The conjugates of Compound 42 exhibit equal or greater fluorescence than the conjugates of CY-5 dye at similar degrees of substitution.

Example 50. Labeling β-galactosidase with a thiol-reactive dye.

A solution of β-galactosidase, a protein rich in free thiol groups, is prepared in PBS (2.0 mg in 400 μL). The protein solution is then treated with a 20 mg/mL solution of Compound 43 in DMF. Unreacted dye is removed on a spin column. The degree of substitution by the dye is estimated using the extinction coefficient of the free dye. The protein concentration is estimated from the absorbance at 280 nm, corrected for the absorbance of Compound 43 at that wavelength.

Example 51. Fluorescence energy transfer in a sulfonated-rhodamine conjugate of R-phycoerythrin.

An R-phycoerythrin conjugate, prepared as in Example 47, is excited at 488 nm and the fluorescence emission is compared to that of unmodified R-phycoerythrin excited at the same wavelength. Highly efficient energy transfer occurs from the protein to the fluorescent dye. A conjugate of this complex with streptavidin is prepared essentially as

described by Haugland (METH. MOL. BIOL. **45**, 205 (1995), *supra*). This conjugate retains the energy transfer properties and is useful for cell staining in flow cytometers that utilize the argon-ion laser for excitation.

Example 52. Labeling and use of a wheat germ agglutinin dye-conjugate.

Wheat germ agglutinin (100 mg, EY Laboratories) is dissolved in 5 mL $NaHCO_3$, pH 8.3, containing 9 mg *N*-acetylglucosamine. To this is added 9 mg of Compound 42. After 1 hour the solution is purified by gel filtration. A degree of substitution of 2–3 dyes per molecule is determined from the absorption at 633 nm.

A 1 mg/mL stock solution of the resulting wheat germ agglutinin (WGA) conjugate (Compound 42) is prepared in 0.1 M sodium bicarbonate ~pH 8. *Staphylococcus aureus* are cultured for 17 hours at 30 °C in TSB broth. Equal volumes of the TSB culture and a BSA solution (0.25% BSA + 0.85% NaCl sterile filtered through 0.2 μM filter) are incubated at room temperature for 15 minutes. The BSA-bacterial suspension (200 μL) is centrifuged for 2 minutes at 350 × g, capturing the bacteria on a filter membrane. The cells are resuspended in 90 μL of BSA solution and 10 μL of stain is added for 15 minutes. Following centrifugation, the bacteria are resuspended in BSA solution, and an aliquot is trapped between a slide and a glass coverslip.

The bacteria are observed on a Nikon Diaphot epi-fluorescence microscope using a fluorescein band pass filter set. Images are acquired using the Star-1 cooled CCD camera and the software package supplied with the camera is used for data analysis. Two images are collected for each stain, each image having a 2 sec. exposure time. When used according to Sizemore et al. (U.S. Patent No. 5,137,810) the conjugate can distinguish between Gram positive and Gram negative bacteria.

Example 53. Simultaneous labeling of actin and tubulin in cultured mammalian cells.

Bovine pulmonary artery cells (BPAEC) are grown to 30-50% confluence on glass. The cells are fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 6% bovine serum albumin (BSA). All cells are incubated with mouse monoclonal anti-α-tubulin for 60 min.

A cell sample is labeled with a monoclonal mouse anti-tubulin (Molecular Probes, Inc.) and a goat anti-mouse IgG conjugate of ALEXA FLUOR 488 (Molecular Probes, Inc., Eugene, Oregon) for 30 min, washed, and then incubated with the phalloidin dye-conjugate of Example 43 for an additional 30 min. The cells are rinsed with blocking buffer and mounted in phosphate-buffered saline (PBS) pH 7.4. The stained cells display microtubules decorated with green fluorescence and actin filaments decorated with red fluorescence.

Example 54. Utility of protein dye-conjugates as immunoreagents and resistance to photobleaching.

Goat anti-Mouse IgG conjugates of Compound 42 are prepared with degrees of substitution of approximately 2–4 (as in Example 47). CY5-labelled goat anti-mouse IgG is purchased from Jackson Immunoresearch. HEp-2 cell slides from INOVA (San Diego, CA) are hydrated in 1% bovine serum albumin (BSA) in PBS for 30 minutes. The slide is drained, human anti-nuclear antibody is applied, the slide is incubated 30 min and rinsed in PBS. Mouse anti-human antibody is applied, the slide is incubated 30 min and rinsed in PBS. The fluorescent anti-mouse antibody conjugate of choice is applied as a 5 μg/mL solution, diluted in 1% BSA/PBS. After 30 minutes the slides are rinsed in PBS, then in 50 mM Tris pH 8.0, mounted in 50 mM Tris pH 8.0, and viewed through an appropriate filter. All samples give predominantly nuclear staining. Quantitative intensity measurements permit comparison of dyes. Similar results are obtained using a biotinylated anti-mouse preparation and fluorescent streptavidin conjugates.

For photobleaching measurements, one image of the slide is acquired every 5 seconds for 100 seconds with continuous illumination. Three fields of cells are bleached, and the photobleaching values are normalized and averaged. The antibody conjugates of Compound 42 are significantly more photostable than those of CY-5 dye (Figure 2).

Example 55. Preparation and use of a fluorescent α-bungarotoxin dye-conjugate.

α-Bungarotoxin (1 mg) in 25 μL 0.1 M $NaHCO_3$ is treated with 1.5 equivalents of Compound 42 at room temperature for 2 hours. The product is purified by size exclusion, by ion exchange chromatography, and finally by reverse phase HPLC. Staining of acetylcholine receptors and detection of their resulting fluorescence is comparable to that obtained with TEXAS RED dye-conjugated α-bungarotoxin.

Example 56. Preparation of aminodextran dye-conjugates.

70,000 MW aminodextran (50 mg) derivatized with an average of 13 amino groups, is dissolved at 10 mg/mL in 0.1 M $NaHCO_3$. Compound 46 is added so as to give dye/dextran ratio of ~12. After 6 hours the conjugate is purified on SEPHADEX G-50, eluting with water. Typically 4-6 moles of dye are conjugated to 70,000 g dextran.

Example 57. Preparation of fluorescent-dye labeled microspheres.

Uniform microspheres are conjugated to the dyes of the invention by one of five methods. In Method A, 1.0 μm amine-derivatized polystyrene microspheres are suspended at ~2% solids in 100 mM $NaHCO_3$, pH 8.3 and treated with 2 mg/mL of an amine-reactive dye. After 1 hour the microspheres are centrifuged and washed with buffer.

In Method B, carboxylate-modified microspheres are suspended in a solution of a protein that has been conjugated to a dye of the invention. The protein is passively adsorbed on the microspheres, and excess protein is removed by centrifugation and washing. Microparticles of a size that cannot be centrifuged are separated from excess protein by dialysis through a semi-permeable membrane with a high MW cutoff or by gel filtration chromatography.

In Method C the protein is covalently coupled through its amine residues to the carboxylate groups of the polymer using ethyl 3-(dimethylaminopropyl)carbodiimide

(EDAC).

In Method D, biotinylated microspheres are treated with a streptavidin, avidin or anti-biotin conjugate of a dye of the invention, and the conjugates are isolated as in Method B.

In Method E, the microparticle is placed in a nonpolar solution of the desired dye. The microparticle swells in the presence of the organic solvent, and the dye is able to permeate the interior of the microparticle. Upon removing the solvent, the microparticle returns to its normal size, trapping the dye within the microparticle

For example, to a stirred 100 mL suspension of carboxyate-modified latex (Interfacial Dynamics Corp., Portland, OR) that is 4.2% solids is added 50 mL methanol. A dye solution is prepared that is 25-50 mg desired dye(s), 7.5 mL methylene chloride and 17.5 mL ethanol. The dye solution is added to the latex suspension at a low flow rate (~ 6 mL/hr), with stirring, using a syringe pump fitted with a Teflon delivery tube. After addition is complete, the organic solvents are removed under reduced pressure, and the aqueous suspension of dyed latex is filtered through glass wool to remove any additional debris. The microparticles are dialyzed in E-pure water (25 mm tubing, MW cutoff 12,000-14,000) until no more free dye is removed from the particles. The fluorescent latex suspension is filtered again through glass wool and then sonicated in a bath sonicator for 5 minutes to ensure monodispersity.

The larger particles can be analyzed for uniformity of staining and brightness using flow cytometry. The microspheres can be further coupled to proteins, oligonucleotides, haptens and other biomolecules for assays using methods well know in the art.

Example 58. Preparation of fluorescent liposomes using the dyes of the invention.

Selected dyes of the invention are sufficiently water soluble to be incorporated into the interior of liposomes by methods well known in the art (J. BIOL. CHEM. 257, 13892

(1982) and PROC. NATL. ACAD. SCI. USA 75, 4194 (1978)). Alternatively, liposomes containing dyes of the invention having a lipophilic substituent (e.g. alkyl having 11-22 carbons), within their membranes are prepared by co-dissolving the fluorescent lipid and the unlabeled phospholipid(s) that make up the liposome before forming the liposome dispersion essentially as described by Szoka, Jr. et al. (ANN. REV. BIOPHYS. BIOENG. **9**, 467 (1980)).

Example 59. Preparation of fluorescent dye-conjugates of bacteria.

Heat-killed *Escherichia coli* are suspended at 10 mg/mL in pH 8–9 buffer then incubated with 0.5–1.0 mg/mL of an amine-reactive dye, such as Compound 42. After 30–60 minutes the labeled bacteria are centrifuged and washed several times with buffer to remove any unconjugated dye. Labeled bacteria that are opsonized are taken up by macrophage, as determined by flow cytometry.

Example 60. Preparation of a nucleotide dye-conjugate.

To 2 mg of 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate (Sigma Chemical) in 100 μL water is added Compound 42 in 100 μL DMF and 5 μL triethylamine. After 3 hours, the solution is evaporated and the residue is purified by HPLC. The product fractions are lyophilized to give the red fluorescent nucleotide conjugate.

Alternatively fluorescent dye-conjugates of deoxyuridine 5′-triphosphate are prepared from 5-(3-amino-1-propynyl)-2′-deoxyuridine 5′-triphosphate (as described in Hobbs, Jr. et al, *supra*).

Example 61. Preparation of an oligonucleotide dye-conjugate.

A 5′-amine-modified, 18-base M13 primer sequence (~100 μg) is dissolved in 4 μL 10 mMTris-HCl, pH 8, 1 mM EDTA. To this is added 250 μg of Compound 42 in 100 μL 0.1 M sodium borate, pH 8.5. After 16 hours, 10 μL of 5 M NaCl and 3 volumes of cold ethanol are added. The mixture is cooled to -20 °C, centrifuged, the supernatant is decanted, the pellet

is rinsed with ethanol and then dissolved in 100 μL H_2O. The labeled oligonucleotide is purified by HPLC on a 300A C8 reverse-phase column using a ramp gradient of 0.1 M triethylammonium acetate (pH ~7) and acetonitrile (5→95% over 30 min). The desired peak is collected and evaporated to give the fluorescent oligonucleotide.

Example 62. Preparing DNA hybridization probes using fluorescent nucleotide dye-conjugates.

For each labeling reaction, a microfuge tube containing about 1 μg of a ~700 bp Hind III – Bgl II fragment of the *E. coli lacZ* structural gene is heated for ~10 minutes at 95 °C to fully separate the strands. The DNA is cooled on ice. A 2 μL of a 2 mg/mL mixture of random sequence hexanucleotides in 0.5 M Tris-HCl, pH 7.2, 0.1 M $MgCl_2$, 1 mM dithiothreitol is added, followed by 2 μL of a dNTP labeling mixture (1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.65 mM dTTP and 0.35 mM fluorescent-labeled dUTP (as prepared in Example 60). Sterile distilled, deionized water is added to bring the total volume to 19 μL. 1 μL Klenow DNA polymerase (2 units/μL) is added. The samples are incubated 1 hr at 37 °C. The reactions are stopped with 2 μL of 0.2 M EDTA, pH 8.0. The labeled DNA is precipitated with 2.5 μL of 4 M LiCl and 75 μL of -20°C ethanol. After 2 hours at -20 °C the precipitated nucleic acids are centrifuged at 12,000 rpm. The pellets are washed with cold 70% ethanol, then cold 100% ethanol. The pellets are dried and dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. A portion of each sample is analyzed by gel electrophoresis on a 1% agarose minigel under standard conditions. The labeled DNA products are suitable for *in situ* hybridization experiments for the detection of RNA or DNA, such as is associated with the *E. coli lacZ* gene in cells or tissues.

Example 63. Incorporation of fluorescent nucleotide conjugates into DNA amplification products.

A DNA amplification reaction is prepared as follows: 1 μL each of 20 μM solutions of two oligonucleotide primers that hybridize to the human β-actin gene are added to a labeling reaction containing 5 μL DNA template (100 pmol of a plasmid containing the entire gene), 5 μL 10X reaction buffer (100 mM Tris, pH 8.3, 500 mM KCl), 2.5 μL 1 mM

fluorescent-labeled dUTP (as prepared in Example 60), 1 μL 10 mM dATP, 1 μL 10 mM dCTP, 1 μL 10 mM dGTP, 1.5 μL 5 mM dTTP, 3 μL 25 mM $MgCl_2$, and 28 μL distilled, deionized water. The sample is transferred to a thermocycler and processed as follows: one cycle, 94 °C, 2.5 minutes; 30 cycles, 94 °C, 1 minute, 50 °C, 1 minute, 72 °C, 1 minute; one cycle, 72 °C, 5 minutes; then 4 °C overnight. An aliquot of the sample is mixed with an equal volume of 10% glycerol, loaded onto a 0.9% agarose minigel and electrophoresed. Fluorescent bands of the expected size are visible when the gel is illuminated with 300-nm ultraviolet light.

Example 64. *In situ* hybridization of an RNA probe.

Mouse fibroblasts are fixed and prepared for mRNA *in situ* hybridization using standard procedures. A dye-labeled RNA probe is prepared by *in vitro* transcription of a plasmid containing the mouse actin structural gene cloned downstream of a phage T3 RNA polymerase promoter. Labeling reactions consist of combining 2 μL DNA template (1 μg DNA), 1 μL each of 10 mM ATP, CTP and GTP, 0.75 μL 10 mM UTP, 2.5 μL 1 mM fluorescent-labeled UTP, 2 μL 10X transcription buffer (400 mM Tris, pH 8.0, 100 mM $MgCl_2$, 20 mM spermidine, 100 mM NaCl), 1 μL T3 RNA polymerase (40 units/μL), 1 μL 2 mg/mL BSA, and 8.75 μL water. Reactions are incubated at 37 °C for two hours.

The DNA template is removed by treatment with 20 units DNase I for 15 minutes, at 37 °C. The RNA transcript is purified by extraction with an equal volume of phenol:chloroform, 1:1, then by chromatography on SEPHADEX G50. Labeled RNA is denatured for 5 minutes at 50 °C, then hybridized to cellular preparations using standard procedures. When preparations are washed and viewed through an appropriate filter set on a fluorescence microscope (excitation 610 ± 10 nm; emission 670 ± 20 nm), cells expressing actin mRNA show bright red fluorescence.

Example 65. Preparing DNA hybridization probes using fluorescent platinum dye-compounds.

A fluorescent platinum complex is prepared from a compound of the invention by adapting the methods provided in U.S. Patent No. 5,714,327 to Houthoff et. al. (1998). For each labeling reaction, a microfuge tube containing 1 μg of pUC1.77 plasmid DNA containing a chromosome 1 human α-satellite probe (DNase treated to a fragment size between 500-1000bp) in 5mM Tris, pH 8, 1mM EDTA, is heated for ~10 minutes at 95 °C to fully denature the DNA. The DNA is cooled on ice. 1 μL of a 1 mg/mL solution of the prepared platinum complex is added, followed by the addition of 5 mM Tris, pH 8, 1 mM EDTA to bring the total volume to 25 μL. The samples are incubated 15 minutes at 80 °C. The reactions are stopped on ice. The labeled DNA is purified on a Bio-Rad Micro Bio-Spin P-30 Tris Chromatography Column. The labeled DNA products are suitable for *in situ* hybridization experiments.

Example 66. Preparing DNA hybridization probes using amine-modified DNA an amine-reactive dye of the invention.

Nick translation is performed using pUC1.77 plasmid DNA containing a chromosome 1 human α-satellite probe. To a microcentrifuge tube is added, in the following order: 23.5 μL H_2O, 5 μL 10X Nick Translation buffer (0.5 M Tris-HCL, 50 mM $MgCl_2$, 0.5mg/ml BSA, pH 7.8), 5 μL 0.1 M DTT, 4 μL d(GAC)TP mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP), 1 μL 0.5 mM dTTP, 4 μL 0.5 mM aminoallyl-dUTP, 1 μL 1 μg/μL template DNA, 5 μL DNase I (1 μg/mL, 2000 Kunitz units/mg), 1.5 μL DNA polymerase I (10 U/μL). The tube is incubated 2 hours at 15 °C, then brought to a final volume of 100 μL with H_2O. The amine-modified DNA is purified using a QIAQUICK PCR purification Kit (Qiagen) with the following modifications to purify the DNA from the enzyme and amine-containing compounds: 75% EtOH is substituted for the wash buffer, H_2O is substituted for the elution buffer, and elution is performed twice for 5 minutes each. The DNA is precipitated by adding 1/10 volume 3M sodium acetate and 2.5 volumes 100% EtOH, incubated at -70 °C for 30 minutes, centrifuged for 15 minutes, and washed with 70% EtOH.

The amine-modified DNA is resuspended in 5 μL H_2O. To the solution is added 3 μL 25mg/ml sodium bicarbonate and 50 μg Compound 42 in 5 μL DMF. The reaction is

incubated for 1 hour at room temperature in the dark. 90 μL H_2O is added to the reaction and it is purified using a QIAQUICK PCR purification kit (QIAGEN), with the following modifications: three washes are performed with 75% EtOH and three elutions of 5 minutes each with the QIAGEN elution buffer. The DNA is precipitated as before. The labeled DNA products are suitable for *in situ* hybridization experiments.

Example 67. Discrimination of live and dead cells using the dyes of the invention.

Selected dyes of the invention are highly polar, and therefore relatively impermeable to the membranes of live cells. These dyes can therefore be used to discriminate cells that have intact versus compromised cell membranes in a single-color assay as follows:

Mouse monocyte-macrophage, Abelson Leukemia Virus Transformed (RAW264.7) cells are trypsinized and washed with phosphate buffered saline (PBS), pH 7.2. Approximately 8–10 million cells suspended in 180 μL of PBS, pH 7.2 are placed in a glass test tube and heated in a water bath at 50 ºC for 20 minutes to kill a fraction of the cells. Approximately 60 μL (2–3 million cells) of the cell suspension is added to 940 μL of PBS, pH 7.2, followed by 0.1 μL of a 1 mg/mL solution of Compound 42 in DMSO. The mixture is incubated on ice for 30 minutes and washed twice with PBS, followed by addition of 200 μL of PBS, pH 7.2. An identical aliquot of cells is treated with 2 μL of a 150 μM solution of propidium iodide in water (as a control for dead cells). Analysis of the cell suspension using flow cytometry shows that populations of dead cells stained by Compound 42 and those stained by propidium iodide are very similar.

Example 68. Neuronal tracing using a hydrazide-labeled fluorophore.

Neurons from zebrafish embryos are microinjected with Compound 44, using standard methods as described by Blankenfeld et al. (J. NEUROSCI. METH. **36**, 309 (1991)). The neurons rapidly fill with the dye throughout their volume and their red fluorescence is readily observable. The staining is fixable in the cells using formaldehyde and standard fixing methods.